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Effect of dietary seaweed supplementation on growth performance, antioxidant and immune responses in European Seabass (*Dicentrarchus labrax)* subjected to rearing temperature and salinity oscillations

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Abstract

Aquaculture is considered to be the fastest growing animal food-producing sector. The increase in world fish *per capita* consumption, followed by the growing percentage of the supply that derives from aquaculture, leads to high demand for more efficient fish production. However, the intensification of aquaculture production brought some constraints. In intensive aquaculture, fish are exposed to several, biological, chemical and/or physical stressors. The decrease in water quality may reduce fish performance and welfare, also increasing the risks of diseases outbreaks. Fungi, bacterial, and viral diseases, as well as suboptimal abiotic conditions are a common cause of growth inhibition and mass mortalities.

Until recently, intensive aquaculture production relied on the use of antibiotics for treatment. However, strict regulations are limiting the over-use of antibiotics aiming at preventing antibiotic resistant bacteria and minimizing consumer's health risk. Vaccination is one of the most effective methods of controlling disease but the development of vaccines against intracellular pathogens has not been successful so far.

The use of immunostimulants appears to be an alternative to antibiotics and vaccines. These components increase resistance to infectious disease and immune competency, not by enhancing specific responses, but by enhancing non-specific mechanisms. The use of immunostimulants, in addition to chemotherapeutic agents and vaccines, is now widely accepted by fish farmers. Still, many questions remain unanswered about the efficiency of immunostimulants, such as whether they can protect against all infectious diseases. Dietary supplementation with immunostimulant substances seems to be a logical step to prevent fish diseases or to lower stress levels, particularly when fish are subjected to environmental oscillations, such as temperature and salinity.

This study evaluated the effect of dietary seaweed mix supplementation in European seabass (*Dicentrarchus labrax*) subjected to rearing temperature and salinity oscillations. Two experimental diets where formulated, a control and a supplemented one, with 7.5% seaweed mix (2.5% *Fucus*, 2.5% *Gracilaria* and 2.5% *Ulva*). Three trials were conducted, where salinity and temperature oscillated separately (trials 1 and 2) or simultaneously (trial 3), simulating natural variations in an aquaculture farm. Growth performance, immune and oxidative stress responses were analyzed.

In trial 1 (salinity), fish fed diets supplemented with seaweed showed lower growth performance than the control diet ($P<0.05$). Trial 2 (temperature), the seaweed diet increased lysozyme activity ($P<0.05$). In trial 3 (simultaneous oscillation of salinity and

temperature), dietary seaweed supplementation did not affect the parameters analyzed. Nevertheless, environmental oscillation significantly affected seabass growth rates (Daily Growth Index), peroxidase activity, and some oxidative stress indicators (total glutathione and oxidized glutathione) ($P<0.05$). Overall, the current study showed that dietary seaweed supplementation may improve immune defenses when seabass is subjected to temperature oscillations.

Keywords: *Dicentrarchus labrax;* European seabass; innate immune system; oxidative stress; seaweeds; supplementation.

Resumo

A aquacultura é considerada o sector de produção animal com maior crescimento. O aumento do consumo de peixe *per capita*, seguido do crescimento percentual da procura de derivados da aquacultura, leva a uma maior necessidade da otimização da produção de pescado. No entanto, a subsequente intensificação trouxe alguns constrangimentos. Em aquacultura intensiva, os peixes estão sujeitos a vários fatores de estresse, sendo eles biológicos, químicos e/ou físicos. A diminuição da qualidade da água pode reduzir o desempenho do peixe e o seu bem-estar, aumentando também os riscos de surtos de doenças. Doenças provocadas por fungos, bactérias e vírus, bem como as condições abióticas sub-ótimas, podem levar à diminuição de crescimento e mortalidades em massa.

Até recentemente, a produção em aquacultura intensiva baseou-se no uso de antibióticos para o tratamento de doenças. No entanto, a crescente restrição regulamentar visa limitar o uso excessivo de antibióticos, tendo como objetivo a prevenção de bactérias resistentes a antibióticos e minimizar o risco para a saúde do consumidor. A vacinação é um dos métodos mais eficazes de controlo de doenças, mas o desenvolvimento de vacinas contra agentes patogénicos intracelulares não tem sido bem-sucedida até à data, não sendo, portanto, uma solução única.

A utilização de imunoestimulantes parece ser uma alternativa aos antibióticos e vacinas. Estes ajudam a aumentar a resistência a doenças infeciosas e aumentar a imunocompetência, não por promover as respostas específicas, mas através do reforço de mecanismos não específicos. A utilização de imunoestimulantes, além de agentes quimioterápicos e vacinas, é hoje amplamente aceite pelos piscicultores. Ainda assim, muitas questões permanecem sobre a eficácia dos imunoestimulantes, se eles são capazes de oferecer proteção contra todas as doenças infeciosas. A suplementação alimentar com substâncias imunoestimulantes parece ser um passo lógico para evitar doenças nos peixes ou para baixar os seus níveis de estresse, particularmente quando submetidos a oscilações abióticas, tais como temperatura e salinidade.

Neste estudo, foram avaliados os efeitos da suplementação alimentar com algas em robalo europeu (*Dicentrarchus labrax*), quando submetido a variações de temperatura e salinidade. Foram formuladas duas dietas experimentais, uma controlo e uma suplementada com 7.5% de mistura de macroalgas (2.5% *Fucus*, 2.5% *Gracilaria* e 2.5% *Ulva*). Foram realizados três ensaios, onde a salinidade e temperatura oscilaram, separadamente ou em conjunto, definidas tendo em conta variações reais numa

aquacultura. O desempenho de crescimento, eficácia de utilização do alimento, parâmetros imunes e estresse oxidativo foram avaliados.

No ensaio 1 (salinidade), apenas o crescimento foi afetado pela suplementação alimentar de macroalgas, sendo inferior ao da dieta controlo ($P<0.05$). No ensaio 2 (temperatura) verificaram-se diferenças significativas na atividade da lisozima, sendo esta maior na dieta de suplementação com macroalgas ($P<0.05$). Nenhum dos parâmetros avaliados no ensaio 3 (oscilações de salinidade e temperatura) foram afetados pelo tratamento com macroalgas. No entanto, neste ensaio, os grupos fixos e oscilatórios mostraram diferenças significativas no índice de crescimento diário (DGI), atividade da peroxidase e, relativamente ao estresse oxidativo, em glutationa total (TG) e glutationa oxidada (GSSG) ($P<0.05$). De uma forma geral, estes resultados mostram que a suplementação com algas pode, sob oscilações de temperatura, ser eficaz na melhoria da resposta imune do robalo.

Palavras-chave: *Dicentrarchus labrax;* robalo; sistema imune inato; stress oxidativo; macroalgas; suplementação.

Index

Tables list

Figures list

Abbreviations

ACH50 - Alternative complement pathway

ANFs - Anti-nutritional factors

CAT - Catalase

DGI - Daily growth index

FBW - Final body weight

FCR - Feed conversion ratio

GPx - Glutathione peroxidase

GR - Glutathione reductase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

GST - Glutathione S-transferase

IBW - Initial body weight

ICBAS - Instituto de Ciências Biomédicas Abel Salazar

IMTA - Integrated multitrofic aquaculture

INE - Instituto Nacional de estatistica

IPMA - Instituto Português do Mar e Atmosfera

LPO - Lipid peroxidation

MDA - Malondialdehyde

MDA - Malondialdehyde

NK - Natural killer

PER - Protein efficiency ratio

PMS - Post-mitochondrial supernatant

ROS - Reactive oxygen species

SOD - Superoxide dismutase

TBARS - Thiobarbituric acid reactive substances

TG - Total glutathione

TMB - Tetramethylbenzidine

TNB - Nitrobenzoic acid

USD – United States Dollar

Introduction

CURRENT STATE OF AQUACULTURE

Terrestrial food sources have evolved for thousands of years from hunter-gathering activities into agriculture, but only recently the capture of wild fish began to transition to aquaculture (FAO, 2016). From 1980, the total of capture fisheries has stagnated at 90 million tons and aquaculture production has become the main responsible for most of the fish supply for human consumption. Indeed, and for the first time ever, in 2014, the contribution from the aquaculture sector of fish supply for human consumption has surpassed that of wild-caught fish (FAO, 2016). Also, the increase in world *per capita* fish consumption, grew from 9.9 kg in 1960 to 19.7 kg in 2013, and overpassed 20 kg by 2014 (FAO, 2016), emphasizing the importance of this industry for the future of seafood for human consumption. Most recent data shows that aquaculture is considered to be the fastest growing animal food-producing sector (FAO, 2016) where feeding is accounted as the major constraint in many developing countries (FAO, 2016). Even though the demand for alternative ingredients is increasing, feed ingredients from marine origin, such as fishmeal and fish oils, are still the preferential ingredients for fish feeds, due to its nutritional quality, including essential amino acids and fatty acids profiles. According to FAO (2016), a recent study emphasizes the need to improve and optimize feed production and on-farm feed management practices in aquaculture. Providing farmers well-balanced feeds at cost effective prices is a necessity to reach a profitable production. Therefore, improvements to the quality and preparation of feeds should help productivity and cut costs down.

The need to compensate the stagnation of fishery production and the increase in food demand has caused an expansion of aquaculture production through area enlargement, intensification and diversification. However, the intensification of aquaculture production brought some constraints. In intensive aquaculture, fish are exposed to several stressors from biological, chemical and/or physical origin. Also, deterioration of the wellbeing of farmed fish is directly correlated with overcrowding conditions (Vadstein, 1997). Water quality in aquaculture systems is affected by high density production and the decrease in water quality may reduce fish performance and welfare, increasing the risks of diseases outbreaks (Vadstein, 1997; Sakai, 1999). Previous studies showed that crowding conditions caused an increase in cortisol levels in *Solea senegalensis*, *Sparus aurata*, and salmonid fishes (Pickering and Pottinger, 1989; Pickering, 1992; Montero *et al.*, 1999; Sangiao-Alvarellos *et al.*, 2005; Costas *et al.*, 2008). High densities also negatively affect growth, feed intake and/or feed conversion ratios (Pichavant *et al.*, 2001; Ellis *et al.*, 2002; Person-Le Ruyet *et al.*, 2003; Lemarie *et al.*, 2004; Björnsson and Ólafsdóttir, 2006).

Additionally, suboptimal biotic (e.g. fungi, bacterial, and viral diseases) and abiotic (e.g. temperature, salinity, dissolved oxygen) conditions are common causes of growth inhibition and mass mortalities. Until recently, intensive aquaculture production relied on the use of antibiotics for treatment (Bagni *et al.*, 2000). However, current strict regulations are limiting the use of anti-biotics aiming to prevent antibiotic resistant bacteria and to minimize consumer's health risk (Cook *et al.*, 2003). Chemical therapy, for example, is used in aquaculture even though it represents a potential environmental hazard and could also have negative effects on the fillet quality (Bagni *et al.*, 2000). Multiple chemotherapeutics were used in the past 20 years to treat bacterial infections in fish cultures, but the incidence of drug-resistant bacteria has risen to be a major problem in fish culture (Aoki, 1992). Vaccines are used to reduce the risk of diseases, but the effectiveness is limited to few biological agents (Sakai, 1999). For instance, vaccines against intracellular pathogens have not been successful so far, and so, they are not a solution that covers all diseases (Sakai, 1999). The use of immunostimulants, in addition to chemotherapeutic agents and vaccines, is now widely accepted by fish farmers (Bricknell and Dalmo, 2005), appearing to be an effective alternative and/or complement to traditional methods. Immunostimulants increase resistance to infectious diseases and increase immunocompetency, not by enhancing specific responses, but by enhancing non-specific mechanisms. This response is usually of short duration and no memory component is involved (Sakai, 1999). However, many questions remain about the efficacy of immunostimulants such as, whether they can protect against all infectious diseases (Sakai, 1999). Other approaches, such as dietary supplementation seem to be a logical step to prevent fish disease or lower stress levels, particularly when subjected to abiotic oscillations, such as temperature and salinity.

EUROPEAN SEABASS (*Dicentrarchus labrax*, Linnaeus, 1758)

European Seabass (*Dicentrarchus labrax*) was chosen as a model species. It belongs to the Chordata Phylum, Actinopterygii Class, Perciformes Order, Percoidei Suborder, Moronidae Family and Dicentrarchus Genus. It possesses an elongated body, males can reach 1 meter length, weight up to 12 kg and live up to 3 decades. Adults have a bright silver tone throughout the body and juveniles tend to be slightly darker with black spots over the back (Fishbase, 2016). European seabass can be found from Eastern Atlantic to Morocco, Canary Islands, Senegal, Mediterranean and Black sea. It is a gonochoristic species, with both sexes found in separate individuals. Spawning occurs in batches and the males and females gather in large groups at open sea. There is only one breeding season per year, which takes place in the winter (December to March) in the Mediterranean population, and up to June in Atlantic populations. Young larvae are then passively transported to nursery areas, such as shallow water lagoons and estuaries. These remain in creeks, estuaries, backwaters, and shallow bays through their first and second years, after which they migrate to over wintering areas in deeper waters, returning to larger estuaries in the summer (ICES, 2013). Since it is a euryhaline (3 ‰ to full strength sea water) and eurythermic (5-28 °C) marine species, it can inhabit different environments. It can survive a wide range of salinities, between the sea, the brackish river estuaries and the Mediterranean lagoons (Tsevis *et al.*, 1992); (Dalla Via *et al.*, 1998). European seabass spends the early and more susceptible life stages in European estuaries and coastal areas where the impact of temperature variation is higher in a daily and seasonal basis, in addition to the increasing temperature due to global warming. It is a carnivore fish that feeds mainly on crustaceans, mollusks and other fish. Adults tend to wander often alone, predating fish, while juveniles form shoal groups in search for small crustaceans and mollusks. This fish species has high ability to adapt to different prey, water and bottom compositions (FAO, 2014).

Barnabé (1990) recognized the fish as a new species for cultivation and as an exploited resource in need for conservation and management. Currently, European seabass is a popular and valuable species for commercial aquaculture in the Mediterranean (Conides and Glamuzina, 2006) where 80% of total production is in Europe, providing a revenue of 380 million Euros in 2009, followed by Egypt, with Greece being the biggest exporter (INE, 2013). In 2010, Portugal reported a production of 400 tons, very low compared to its European partners (INE, 2013). Despite the growth in volume, overproduction of this species in the last two decades has led to a decline in sales price, currently at 5 USD/kg. Nevertheless, it continues to be a highly desirable product in the

European markets (FAO, 2012) and improving feeding and production strategies is crucial for the development of the sector.

IMMUNE SYSTEM

Aquaculture's rapid development has increased the frequency of disease outbreaks. It is well documented that the occurrence of a fish disease depends on the balance between host, pathogen, and environment, three factors with continuous interaction (Roberts, 2012). Fish defense system is very similar to that described in mammals (Hoar *et al.*, 1997) and, as in other vertebrates, the innate immune system of fish provides the first defense line. From a phylogenetic point of view, the fish immune system has received interest since fish were the first vertebrates showing the basic aspects of the immune system, such as true lymphocytes, lymphoid tissues, antibody production, T cell cytotoxicity and long term memory (Koumans-van Diepen, 1993). Teleost lymphoid system encompasses thymus, head kidney, spleen and mucosal associated to lymphoid tissue. These, together with cellular components, display humoral and cellular immune responses such as non-specific cell-mediated cytotoxic and microbial killing (Scapigliati *et al.*, 2002).

Regarding cellular defense systems, teleosts possess phagocytic cells similar to macrophages, neutrophils, and natural killer cells (NK), and also T and B lymphocytes. Teleosts have multiple humoral defense components like complement (classical and alternative pathways), lysozyme, natural hemolysin, transferin and c-reactive protein. The presence of cytokines (interferon, interleukin 2, macrophage activating factors) was also reported (Secombes *et al.*, 1996). Immunostimulant treatments using glucan (Yano and Mangindaan, 1989; Chen and Ainsworth, 1992; Jørgensen and Robertsen, 1995; Sakai, 1999), lactoferrin (Sakai *et al.*, 1993), levamisole (Kajita *et al.*, 1990), FK-565 (Kitao *et al.*, 1987), chitin (Sakai *et al.*, 1992), and EF203 (Yoshida *et al.*, 1993) have shown to cause an enhancement activity of phagocytic cells.

Complement system

The complement system is a particularly important first line of innate immune response, and is, therefore, well studied in fish. The complement system consists of a complex enzyme cascade and is composed of several inactive glycoproteins, which can be activated by one of the three known activation routes: classical, alternative, and lectin-mediated. It is composed of more than 35 soluble plasma proteins which are expressed in the liver and released into the plasma. The functions of the complement system include lytic activity (for viruses, bacteria, and parasites) and neutralization of pathogenic exotoxins. The

activated proteins can also provide an alert of potential pathogens present in the host, hence contributing to the degradation of pathogens through the recruitment of immune cells and through opsonization (Boshra *et al.*, 2006). Fish complement system has been showed to be affected by light exposure (Angeles Esteban *et al.*, 2006), environment temperature (Tort et al., 1998), diet (Geay et al., 2011), and more directly correlated, infections (Henry et al., 2009).

Peroxidase

Both neutrophils and macrophages are important innate system components that protect the organism against microbial infection. The functions of both cells include phagocytosis, chemotaxis and bactericidal activity (Katzenback *et al.*, 2012). Upon activation, neutrophils release hydrogen peroxide and myeloperoxidase (EC 1.11.2.2), a heme-containing lysosomal glycoprotein, found predominantly in neutrophils azurophilic granules, and in much lower amounts in monocytes and some tissue macrophages. Myeloperoxidase then catalyzes chloride ions and the hydrogen peroxide to form hypochlorous acid, damaging the invading microorganisms (Klebanoff, 1968). Similar to neutrophils myeloperoxidase, eosinophils produce an eosinophillic peroxidase (EC 1.11.1.7), consisting of a heavier glycosylated chain and a lighter non-glycosylated chain. This enzyme prefers bromide over chloride as substrate, converting it to hypobromite which is toxic to infecting pathogens (Bielek, 1981).

Peroxidases, released from the cytoplasmic granules of phagocytes, take part in the oxidative responses against pathogens. Serum peroxide levels are known to increase in response to infection (Alvarez-Pellitero, 2008). Therefore, peroxidases released into the blood are often used as an indicator of the immunologically active status of circulating leucocytes.

Lysozyme

Lysozyme was the first natural antibiotic isolated from humans in 1922 by Mr. Alexander Fleming (Nakatsuji and Gallo, 2012). As a glycoside hydrolase enzyme, lysozyme (EC 3.2.1.17) can damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. Hence, lysozyme is a useful tool when dealing with pathogens, especially gram positive bacteria, as these have a peptidoglycan outer layer. With leucocyte origin, lysozyme is widely distributed in bacteriophages, microbes, invertebrates and vertebrates and is found in a large variety of animal secretions such as mucus, saliva, and blood (Jollès and Jollès,

1984). Studies have shown that plasmatic lysozyme activity can be modulated by several factors. Lower temperatures have been shown to decrease lysozyme activity in gilthead seabream (*Sparus aurata*) (Tort *et al.*, 1998), but a less linear correlation was found in Nile tilapia (*Oreochromis niloticus*), where a slight increase in temperature revealed to increase lysozyme before a plateau was reached (Dominguez *et al.*, 2005). Also, dietary supplements of probiotic yeast (*Saccharomyces cerevisiae*) have shown to improve lysozyme activity as well (Torrecillas *et al.*, 2007).

OXIDATIVE STRESS

The liver has been the focus of toxicological studies and has, indeed, been shown to be a very sensitive organ to oxidants presence (Ameur *et al.*, 2012). Therefore, it is accepted that the monitoring of antioxidant enzymes activities in the liver may create a good evaluation of the antioxidant state.

Oxidative damage levels and energetic metabolism parameters are among the most commonly used biomarkers of fish health status (Van der Oost *et al.*, 2003). Levels of lipid peroxidation (LPO) are good indicators of reactive oxygen species (ROS) overproduction and induced oxidative damage in cells (Livingstone, 2001; Limón-Pacheco and Gonsebatt, 2009).

A consequence of all aerobic life is the production of potentially harmful, partially reduced species of molecular oxygen radicals (ROS) which occur as a result of normal oxygen metabolism. It has been estimated that about 1 to 3 % of O_2 consumed in animal systems is converted into ROS, generated by physical, chemical, and metabolic processes(Livingstone, 2003). These convert O_2 into reactive oxygen species, such as superoxide, hydroxyl radicals and non-radical hydrogen peroxide or even singlet oxygen. Most ROS production occurs by the auto-oxidation of the mitochondrial electron transport chain, microsomal cytochrome P-450 and flavor-protein reductases (Livingstone, 2003). Although ROS supply the body with humoral innate protection against pathogens, in peroxidases parameter, disturbances in the normal redox state of cells can cause a toxic effect, damaging cell components, including proteins, lipids and DNA. Furthermore, severe oxidative stress can trigger apoptosis, while prolonged extreme oxidative damage may cause tissue necrosis (Zong and Thompson, 2006). These effects depend upon the size of these changes. A cell can be able to overcome small perturbations and regain its original state, without further damage. Only when the production of these aggressions overcome

the organism defense capabilities, does the animal enter the oxidative stress condition (Finkel and Holbrook, 2000).

ROS production can be induced by external factors, with the environmental pollution being one of the most studied. Transition metals (Cu, Fe) represent a major path of environmental oxidation, as they catalyze the production of hydroxyl radicals through Fenton reaction. Other chemicals like biphenyls, quinones, and nitroaromatics can also induce superoxide production by redox cycling (Sevcikova *et al.*, 2011).

Environmental disturbances may induce ROS production since it does not always require physical or chemical hazardous conditions. In aquaculture, several events common to daily maintenance may cause stress to fish, hampering their health and growth performances. Previous studies suggested that oxidative stress in aquaculture fish can be correlated to several types of stressful conditions, such as feed composition (Olsen and Henderson, 1997), feed deprivation (Pascual *et al.*, 2003), crowding (Bagni *et al.*, 2007), hypoxia (Guerriero *et al.*, 2002), acute thermal conditions (Vinagre *et al.*, 2012), and infectious diseases (Ali *et al.*, 2011).

Olsen *et al.* (2002) and Olsen *et al.* (2005) described some negative effects of ROS in the enterocytes lining and junctions of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) submitted to an acute stress, causing a decrease in feed digestion. Vinagre *et al.* (2012) observed that seabass subjected to suboptimal temperatures showed an increased in lipid peroxidation and catalase activity, showing that temperature deviations may cause responses similar to environmental contaminants (Vinagre *et al.*, 2012).

Antioxidant systems can be divided into two categories: enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidases; and free radical scavengers, such as carotenoids, peptides, amino acids and phenolic compounds. These systems are capable of preventing cell membrane damages, enzyme inactivation and nucleic acid alterations caused by ROS (Bragadóttir, 2001).

In a closer description of antioxidant enzymes: CAT (EC 1.11.1.6.) catalyses the decomposition of hydrogen peroxides to water and oxygen and is interpreted as a protector against hydrogen peroxide. Glutathione peroxidase (GPX) (EC 1.11.1.9.), reduces lipid hydro-peroxides to their corresponding alcohols and reduces free hydrogen peroxide to H_2O, using a co-factor, glutathione (GSH). Glutathione reductase (GR) (EC 1.8.1.7), is an enzyme responsible for recovering oxidized glutathione (GSSG) to its useful state (GSH). Glutathione s-transferase (GST) (EC 2.5.1.18), another glutathione (GSH) dependent enzyme, neutralizes xenobiotics, inhibiting these to cause cell damage (Eroglu *et al.*, 2015).

Finally, total glutathione levels (TG), a sum of both GSH and GSSG, a tripeptide that when in its reduced form works as an electron donor to GPX, also as cofactor for GST, in addition to being a direct thiol-based antioxidant. Recent studies suggest that although GSH/GSSG ratio may indicate a prompter antioxidant state, a reduction in total glutathione levels may indicate high utilization of GSH (Eroglu *et al.*, 2015). These molecular parameters constitute the first line of antioxidant enzymatic defenses and are used as biomarkers for oxidative stress condition in a variety of marine and freshwater organisms (Eroglu *et al.*, 2015). Lipid oxidation is the most used approach in free radical research once most aquatic organisms contain lipids with high amounts of polyunsaturated fatty acids (Lushchak and Bagnyukova, 2006). Since lipids are oxidized usually through the formation of peroxides, the process of their formation has been called "lipid peroxidation", and is quantified to determine the degree of damage that may have been caused by ROS over the lipid layers. As this damage is avoided when antioxidant enzymes surpass the oxidants action on cell, this parameter may indicate the organism vulnerability (Lushchak, 2011).

TEMPERATURE

One of the most important environmental factor affecting the biochemical and physiological processes of aquatic organisms is water temperature (Reynolds and Casterlin, 1979). As stated by Sengupta and Garrity (2013) "temperature is an omnipresent physical variable reflecting the rotational, vibrational and translational motion of matter". For what matters in biology, temperature decides a great part of a species success and possible proliferation, mostly due to temperature's ability to mold matter. Temperature dominant modulations can affect proteins and lipids re-conformation, as well as genetic modulation, playing as an on/off trigger in genetic transcription, translation or path activation (Sengupta and Garrity, 2013). A more immediate effect is the increase of kinetic energy in molecules, such as enzymes, since temperature can make molecule collisions more frequent, enabling the enzyme-subtract complex to occur more persistently, hence increasing the reaction rate (Cornish-Bowden, 1979). For this reason, the control of the metabolic activity by temperature is considered key for fish to optimize their ecosystem efficiency (Magnuson *et al.*, 1979). There are also other environmental factors, such as oxygen and salinity, that represent potential determinants of fish metabolic state, being responsible for physiological and/or behavioral changes (Priede, 1985). Temperature variations strongly impact fish due to the lack of body insulation (they are poikilotherms), and the blood-water counter current respiratory system in the gills (Schmidt-Nielsen, 1997; Beitinger *et al.*, 2000). These kind of alterations induce an initial stress phase (thermal stress) with the formation of reactive

oxygen species (ROS), which can damage protein, lipids or DNA, and potentially increase energy demands/requirements (Lushchak and Bagnyukova, 2006).

The adult European seabass can withstand temperatures ranging from 2 to 32 ºC (Barnabé, 1990), with an optimum temperature of 22 °C to 25 °C for best growth and feed utilization performance (Person-Le Ruyet *et al.*, 2004). During autumn, the falling of sea temperature below 10ºC is associated with their migration into deeper, less cold waters (Pickett and Pawson, 1994). Also, temperature has shown to modulate the immune system, protein expressions (Sarropoulou *et al.*, 2010) and antibody response (Cecchini and Saroglia, 2002).

SALINITY

Growth performance of marine species is related to water salinity (Imsland *et al.*, 2007). Indeed, both salinity and temperature have been recognized as two of the most important abiotic factors that can affect the biological metabolism in fish (Mosser and Hettler, 1989; Dalla Via *et al.*, 1998). The energetic costs of ion regulation are generally observed to be lowest in an isosmotic environment, hence it has been hypothesized that this energy saving may be substantial enough to increase growth (Morgan and Iwama, 1991).

Salinity seems to modify multiple aspects related to growth, including standard metabolic rate (Dutil, 1997), feed intake and feed conversion efficiency (Imsland *et al.*, 2007) and the balance of hormones involved in overall metabolism (Bluf, 2001). Still, this response differs depending on the species. Atlantic cod (*Gadus morhua*) (Lambert *et al.*, 1994) and freshwater catfish (*Mystus vittatus*) (Arunachalam and Reddy, 1979) have higher feed conversion efficiency in low salinities (< 14‰), whereas European seabass (Dendrinos and Thorpe, 1985), juvenile flounder (*Platichthys flesus*) (Gutt, 1985), and gilthead seabream (*Sparus aurata*) (Conides *et al.*, 1997) plateau at intermediate salinities (14–28‰). Also, growth rates of European seabass were observed to be higher at salinities above 28-30‰ (Fig. 1) (Conides and Glamuzina, 2006). Seabass of all ages can tolerate freshwater, but it seems that osmoregulation and sexual maturation in brackish waters are incompatible. According to Zanuy and Carrillo (1984), fish kept in brackish water (3‰) managed to complete vitellogenesis and spermatogenesis but cannot release gametes.

Euryhaline fish adaptation to changing salinities induces changes/activation of ion transport mechanisms. This is usually accompanied by changes in oxygen consumption, suggesting variation in the energetic demands for osmoregulation (Sangiao-Alvarellos *et*

al., 2003). Changes in oxygen consumption can lead to variations in whole body metabolism (Sangiao-Alvarellos *et al*., 2003). This metabolic response to different osmotic conditions includes both stress and osmoregulation components, but the energy demands of each process cannot be discerned from the whole fish energy allocation (Sangiao-Alvarellos *et al*., 2003). The weight of environmental salinities on growth rate in fish also needs further study (Boeuf and Payan, 2001).

The tolerance to hypotonic environment is an important physiological ability that is observed in marine fish species in estuarine ecosystems (Day, 1989; Blaber, 1997). There have been many studies on salinity tolerance in salmonids and freshwater fish (Morgan and Iwama, 1991), but very few investigated the tolerance of marine and estuarine species to a range of salinities (Partridge and Jenkins, 2002).

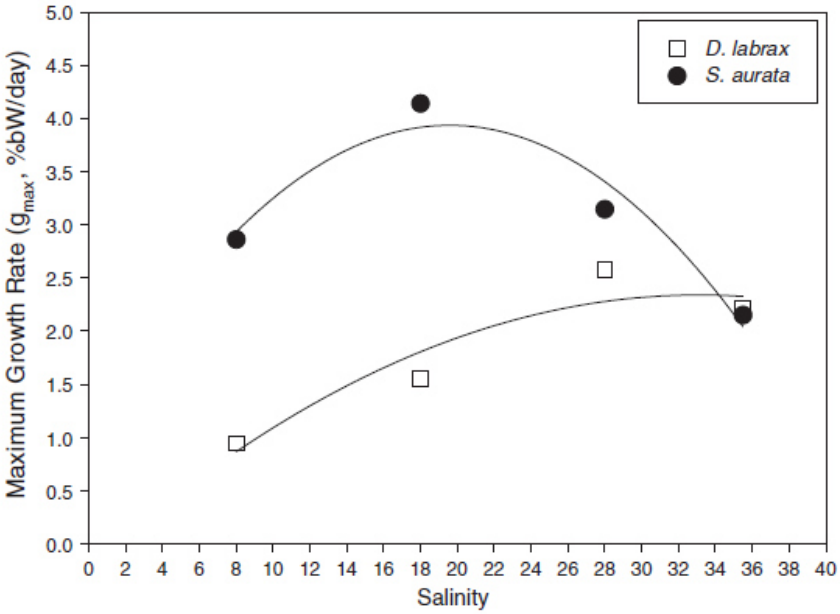

Figure 1: Relationship between maximum growth rates and salinity for European seabass, *Dicentrarchus labrax*, and Gilthead seabream, *Sparus aurata*, juveniles (Conides and Glamuzina, 2006).

Table 1: Maintenance requirement (m) was found to increase with decreasing salinity (Conides and Glamuzina, 2006).

Salinity	Equation	r^2	Std. error (±% bW/day)	m
Dicentrarchus labrax				
Natural	$g=-0.852+1.938 \cdot f-0.307 \cdot f^2$	0.998	0.139	0.475
28‰	$g=-1.427+2.400 \cdot f-0.360 \cdot f^2$	0.999	0.047	0.660
18‰	$g=-1.812+2.108 \cdot f-0.330 \cdot f^2$	0.999	0.106	1.024
8‰	$g=-2.606+1.884 \cdot f-0.250 \cdot f^2$	0.999	0.084	1.824

USE OF IMMUNOSTIMULANTS IN AQUAFEEDS

An immunostimulant could be defined as a "naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens'' (Bricknell and Dalmo, 2005). These compounds increase resistance to infectious diseases by enhancing non-specific defense mechanisms (Sakai, 1999), which may occur before any exposure to a pathogen, improving survival (Sakai, 1999). Immunostimulants can be grouped according to their origin: bacterial (e.g. glucans), algae-derived, animal-derived (e.g. chitosan), nutritional factors, and hormones (cytokines and others) (Sakai, 1999). They can also be used in dietary supplementation due to the relatively low cost of their source ingredients (Bricknell and Dalmo, 2005). Immunostimulants, used as dietary supplements, can improve the innate defenses of fish, providing better resistance to pathogens during periods of greater stress (transport, grading, netting, etc.) (Bagni *et al*., 2000).

Nowadays, the use of dietary immunomodulators is widely accepted in aquaculture. Glucans in salmon diets were one of the first applications of immunostimulants in aquaculture, showing effectiveness in managing disease outbreaks after stressful events (Bricknell and Dalmo, 2005). In hybrid sturgeon (*Acipenser ruthenus* x *A. baerii*) (Jeney and Jeney, 2002) and turbot (*Scophthalmus maximus*) (Low *et al*., 2003), glucans proved to be effective on non-specific defense mechanisms. Long term usage of glucans in seabass has been reported benefits regarding alternative pathways of complement activation and lysozyme activity (Bagni *et al*., 2000), and currently there are commercial diets supplemented with nucleotides that have been proven to reduce sea lice settlement and provide protection against *Aeromonas salmonicida* and *Vibrio anguillarum* infection

(Burrells *et al.*, 2001). Also, the use of immunostimulants in vaccine formulations has given very good antibody responses when used either to replace oil based adjuvants, without the adverse side effects that have been reported for these types of adjuvants, or in addition to them (Ogier de Baulny *et al.*, 1996).

Introduction of dietary immunostimulatory substances in Mediterranean fish farms has been done in an empirical way and there is little information available on their effects. An increase in lysozyme and complement activities was observed by Obach *et al.* (1993) in European seabass juveniles when fed high levels of α-tocopherol. Enhanced phagocytic activity was reported in 1-year-old seabass fed a diet supplemented with levamisole and glucans (Jeney *et al.*, 1994). *In vivo* effects of immunostimulants in fish are described as anti-parasitic (including reduced settlement of sea lice), growth enhancement, increased antibody production following vaccination, increased lysozyme levels and increased survival after bacterial challenges (Bricknell and Dalmo, 2005).

However, the use of immunostimulants can bring unforeseen drawbacks, especially to fish larvae. In Atlantic salmon, when glucans were used as immunostimulants, antigen competition has been observed, suggesting the presence of mechanisms for the induction of tolerance (Killie and Jørgensen, 1994). This has also been studied for other species (Waagbø *et al.*, 1994). Other problems may include those associated with diets' palatability and group hierarchies, where the dominate fish will ingest significantly more feed, thus more immunostimulants than the subordinate fish (Bricknell and Dalmo, 2005).

According to Bricknell and Dalmo 2005, "immunologically mature fish are those who have undergone metamorphosis and have fully developed range of immune responses." Immunostimulant dietary supplementation in mature fishes only contemplates two effective strategies. The continous feed or pulse feed. With continuous feeding, fish can respond by maintaining the immune system up-regulated in high levels until the immunostimulant is withdrawn or, more often, until it induces tolerance. This tolerance is built by the immune system of the fish becoming de-sensitized to the immunostimulant. Continued exposure can, in extreme circumstances, cause the immune response to be suppressed, giving the host a lower level of innate defenses (Bricknell and Dalmo, 2005). To overcome this condition, the most common technique used for adult fish is pulse feeding (Fig. 2). This usually takes 4 to 6 weeks, so as to up-regulate the immune response. Then, the immunostimulant is withdrawn for the same period and the immune system stimulation drops back to the resting level before another dose of the immunostimulant is given (Bricknell and Dalmo, 2005). This forces the immune system of the host to oscillate from resting state to an enhanced response, which provides greater flexibility, since the farmer

can match these states with periods of increase disease risk or stress (Bricknell and Dalmo, 2005). Spring and autumn are seasons of higher stress and disease periods, due to temperature changes, prior to breeding season or to smolts (young salmon or trout) just before sea transfer (Bricknell and Dalmo, 2005).

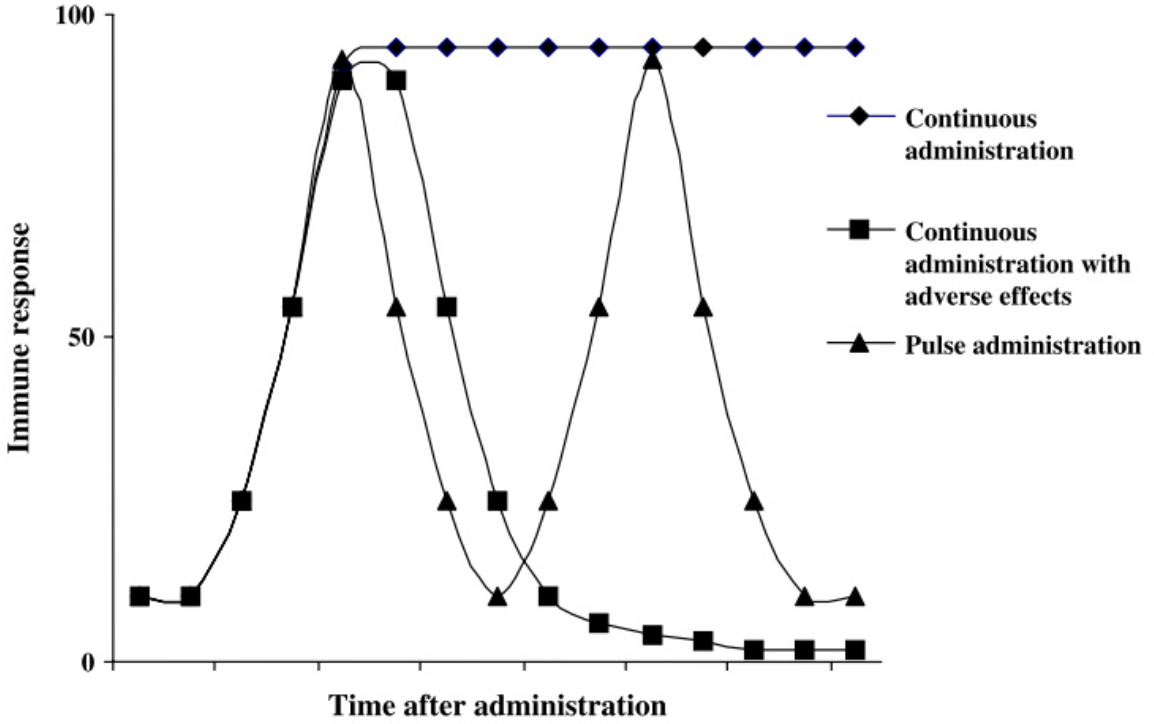

Figure 2: Different outcomes after immunostimulant administration to fish (Bricknell & Dalmo, 2005).

Besides the dietary supplementation, direct injection of immunostimulants has shown to enhance leucocytes function and protection against pathogens (Sakai, 1999). However, this method is labor intensive, time-consuming and impractical for fish weighing less than 15 g. Other methods such as oral administration or immersion should be used. Oral administration has been reported for glucans, EF203, lactoferrin, levamisole and chitosan, enhancing leucocyte function and protection against infectious diseases like furunculosis, vibriosis, streptococcosis (Sakai, 1999). This method allows mass administration, is non-stressful and can be used in fish of all sizes. Alternatively, immersion in a levamisole solution of 10 µg/ml for 24 h showed activated phagocytic activities, chemotactic abilities, and the production of active oxygen in phagocytes and enhanced carp protection against *A. hydrophila* (Baba *et al.*, 1993).

SEAWEEDS

Use of seaweeds in aquafeeds

Over the past decade, extensive efforts have been made worldwide to evaluate a wide range of novel ingredients for fish feeds either as protein sources or as feed additives (Wassef *et al.*, 2013). At least since the 1980's, seaweeds have been studied to be included in fish feeds due to their nutritional quality (Montgomery and Gerking, 1980). Recently, the studies on the dietary seaweed supplementation in fish feed has increased significantly due to their good source of protein (Valente *et al.*, 2006; Dantagnan *et al.*, 2009), amino acids and fatty acids (Wahbeh, 1997; Soler-Vila *et al.*, 2009), vitamins and minerals, and as a coloring agent (Kissil *et al.*, 1992; Soler-Vila *et al.*, 2009). In addition, seaweeds are a source of biologically active compounds with great benefit for both humans and animals (Mustafa and Nakagawa, 1995; Nakagawa, 1997). Several studies reported that supplementing fish with macroalgae led to a considerable enhancement in growth rate, flesh quality and diet utilization (Wassef *et al.*, 2001; Valente *et al.*, 2006; Dantagnan *et al.*, 2009). They were also successfully used as feed attractant for several fish species (Nakajima *et al.*, 1989; Nakajima, 1991; Shields and Lupatsch, 2012).

Many authors have previously shown that different species of seaweed, such as *Ascophyllum nodosum* (Nakagawa *et al.*, 1997), *Gracilaria cornea* and *Gracilaria bursa-pastoris* (Valente *et al.*, 2006), *Porphyra* sp. (Soler-Vila *et al.*, 2009), and *Ulva lactuca* (Wassef *et al.*, 2001) can be used as up to 10% of dietary fishmeal replacement.

Even though seaweeds represent a potential food alternative, they can hold certain substances with some level of toxic and anti-nutrient activity, which can contribute to the reduction of its nutritional value and have a negative impact on fish growth (de Oliveira *et al.*, 2009). Anti-nutrients have been defined as substances which by themselves, or through their metabolic products, interfere with food utilization and can affect the health and production of animals (Makkar, 1993). Anti-nutritional factors, such as lectins, protease inhibitors, goitrogens, allergens, anti-vitamins, saponins, tannins, phytate and toxins, are widely distributed in plants and seaweeds. Also, multiple negative effects caused by these anti-nutritional factors incorporation in fish diets have been reported. Thus, the wide selection of these new food sources needs to previously consider the presence of anti-nutritional factors (Liener, 1994; Francis *et al.*, 2001; Bajpai *et al.*, 2005).

Production of seaweeds

Seaweeds are the main representative of aquatic plant aquaculture, which production is practiced in about 50 countries (FAO, 2016). In the last decades, seaweed production has been increasing but still, commercial scale cultivation remains restricted to a few species (Buschmann *et al.*, 2001; Nagler *et al.*, 2003). Even so, nitrogen-enriched conditions such as aquaculture effluents, are good conditions to enhance seaweed protein content (Lahaye *et al.*, 1995; Pinchetti *et al.*, 1998). Seaweeds produced in integrated multitrophic aquaculture (IMTA) systems have high productivity levels and less variability in protein content than seaweed obtained from natural environments. This is due to the continuous supply of nutrients and the minimum disturbance by grazers or epiphytes (Schuenhoff *et al.*, 2003; Mata *et al.*, 2010; Abreu *et al.*, 2011).

Use of seaweeds as immunostimulants

Besides their high nutritive value, seaweeds are recognized as a valuable source of biologically active substances (Jiménez-Escrig *et al.*, 2011), and their antioxidant and immune properties have been the subject of many studies (Plaza *et al.*, 2008). These bioactive compounds can act as dietary immunostimulants (Peddie *et al.*, 2002; Liao *et al.*, 2003; Díaz-Rosales *et al.*, 2005), consisting in a real asset for controlling disease and improve production (Sakai, 1999).

In *in vitro* conditions, important anti-microbial and anti-viral activities (Hemmingson *et al.*, 2006; Cox *et al.*, 2010; Narasimhan *et al.*, 2013) and efficient antioxidant capacity (Leonard *et al.*, 2011; Narasimhan *et al.*, 2013), have been demonstrated in extracts obtained from representative species of Phaeophyta, Chlorophyte and Rhodophyta and (brown, green, and red seaweed, respectively).

Brown seaweed (Phaeophyceae spp.) have nutraceutical properties due to the presence of compounds, such as laminarin, fucoidan, and polyphenols, which have been shown to improve human health (Plaza *et al.*, 2008). Laminarin is able to modulate the immune response (Neyrinck *et al.*, 2007), and possesses anti-tumor antibodies (Jolles et al., 1963) and anti-apoptosis proprieties (Kim *et al.*, 2012). Fucoidan on the other hand, has a positive effect on extracellular matrix proteins, on cell proliferation (Haroun-Bouhedja *et al.*, 2000; Koyanagi *et al.*, 2003) and can activate apoptosis (Aisa *et al.*, 2005). Fucoidan is also known for its anticoagulant, anti-tumor, anti-thrombosis, anti-inflammatory and anti-viral properties (Berteau and Mulloy, 2003).

Red and green seaweed showed to have significant effects on growth, feed utilization, stress response, physiological condition, body constituents and carcass quality

of cultured fish (Yi and Chang, 1994). According to Xu and Hirata (1990), the use of *Ulva* sp. as a feed additive for black seabream (*Acanthopagrus schlegeli*) and red seabream (*Pagrus major*) has been shown to be beneficial on growth and color. Wassef *et al.* (2005) reported that using *Pterocladia capillacea* (red algae) or *U. lactuca* (green algae) led to enhanced growth performance and feed utilization, improvement in stress response, and survival rate in gilthead seabream. Pham *et al.* (2006), using *Hizikia fusiformis* (brown seaweed) in plaice (*Pleuronectes platessa*) diets was able to improve immune response and to lower mortality rate when compared to fish fed diets without seaweed. Also, supplementing with 5% *Ulva spp.* in diets for red seabream (*Pagrus major*) showed an increased resistance to infection by *Pasteurela piscicida*, without compromising growth (Satoh, 1987). Mustafa and Nakagawa (1995), in a review article, pointed out several beneficial effects of seaweed supplementation to fish feeds, but this area remains rather unexplored by the industry.

During *in vitro* studies, *Ulva* spp. and *Chondrus crispus* extracts and β-glucans have shown to increase respiratory burst and immune system stimulation, through rapid release of ROS and signaling proteins in turbot and Atlantic salmon phagocytes (Dalmo and Seljelid, 1995; Castro *et al.*, 2004). Besides immunocompetency, a positive correlation has been reported between phenolic content and the antioxidant capacity of lipid peroxidation inhibition (Heo *et al.*, 2005). Extract and product administration of red (*Gracilaria folifera*) and brown (*Padina gymnospora* and *Sargassum cinereum*) seaweeds may be effective therapeutic and prophylactic treatments against *Pseudomonas* spp. infection (Thanigaivel *et al.*, 2015). A 2.5% dietary supplementation with *Gracilaria* spp. led to an antioxidant capacity enhancement, improving the innate immune system indicators in European seabass (Peixoto *et al.*, 2016).

Despite the potential of seaweeds, *in vivo* studies are rare in fish and it is vital that additional research is carried out to accurately evaluate the potential of seaweed application as a nutritional tool (Makkar *et al.*, 2016).

Gracilaria vermiculophylla

One of the world's most exploited seaweeds is *Gracilaria* (Pereira *et al.*, 2008), mostly due to the phycocolloid industry, which is a major source of agar (Peng *et al.*, 2009). *Gracilaria vermiculophylla* is a non-indigenous Asian red algae (Nyberg *et al.*, 2009) naturalized in the Ria de Aveiro, Portugal, and is the dominant *Gracilaria* species. It is adapted to shallow soft-bottom bays, lagoons, estuaries, harbors, and inlets (Thomsen and McGlathery, 2007).

It is very resistant to multiple stress factors, such as darkness, sedimentation, desiccation, and different nutrient conditions. It grows under an extensive range of environmental conditions and can be produced throughout the year (Thomsen and McGlathery, 2007; Nyberg *et al.*, 2009; Abreu *et al.*, 2011). They are also efficient biofilters due to their good capacity to remove ammonia and nitrite from the water (Neori *et al.*, 2000; Yokoyama and Ishihi, 2010; Abreu *et al.*, 2011).

Ulva sp.

Ulva sp. is a green algae found in a variety of habitats and on several different subtracts (Schijf and Ebling, 2010). It has a very good vitamin and mineral profile, also rich in glutamic and ascorbic acid, alanine and iron (Briand and Morand, 1997; Ortiz *et al.*, 2006; García-Casal *et al.*, 2007). Even though it does not have the economic value of *Gracilaria* or *Porphyra* species, *Ulva* spp. has been already studied as an ingredient for herbivorous aquatic animals (Dworjanyn *et al.*, 2007) and various fish species, such as European seabass (Valente *et al.*, 2006), common carp (*Cyprinus carpio*) (Diler *et al.*, 2007), Nile tilapia (Güroy *et al.*, 2007; Ergün *et al.*, 2009; Pereira *et al.*, 2012; Marinho *et al.*, 2013), and rainbow trout (Güroy *et al.*, 2011; Güroy *et al.*, 2013). Furthermore, according to Pereira *et al.* (2012), Nile tilapia seems to digest and utilize *G. vermiculophylla*, *Porphyra dioica*, and *Ulva* spp. better than other tested seaweeds (*Sargassum muticum*). The utilization of *Ulva spp.* by European seabass juveniles was evidenced to have no negative consequences on fish performance (Valente *et al.*, 2006).

***Fucus* sp.**

Fucus sp. (Linnaeus, 1753) is a brown macroalgae of the Phylum Ochrophyta, Class Phaeophyceae, Order Fucales and Family Fucaceae. To date, studies on the dietary *Fucus* sp. supplementation in fish feed is very scarce. In a more recent study, however, Peixoto *et al.* (2016) showed that *Fucus* sp. supplementation of up to 7.5% in practical diets for European seabass has no impact on growth performance

Objective of this study

The objective of this study was to evaluate the effects of dietary seaweed supplementation in European seabass, subjected to environmental stress. To do so, fish were exposed to rearing temperature and salinity oscillations and several indicators of growth performance, immune and oxidative stress responses were analyzed.

Materials and methods

The current trial was conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal), as according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

EXPERIMENTAL DIETS

The experimental diets consisted in a control diet (CONTROL) without seaweed supplementation, and a supplemented diet (SEAWEED), to which 7.5% of a seaweed mix was added. The mix was supplied by ALGA+ ® and contained a mixture of *Gracilaria* sp., *Ulva* spp. and *Fucus* sp. Both diets were manufactured by Sparos Ltd., taking into account the species requirements. Diets are isolipidic, isoproteic and isoenergetic (Table 2).

EXPERIMENTAL DESIGN

Three individual trials were performed where salinity, temperature, and a combination of both variations were evaluated. The temperature and salinity values used were selected as most representative according to the annual oscillations in a seabass farm (Materaqua Lda, Ílhavo, Portugal) over the last 3 years (Fig. 3, 4, 5, 6). In trial 1, salinity was used as the variable, and lasted for 56 days with random variations of salinity, changed every 5 days (Table 3). The variations in salinity were obtained by adding sea salt to the water system or replacing sea water with dechlorinated fresh water. In trial 2, water temperature varied every 7 days (Table 3) for 63 days. The variations of temperature were carried out using 2 thermostats heaters (Trixie® – 200 watts) to rise temperature and two water chillers (TECO® TR60) to cool it down. In trial 3 (Table 3), salinity and temperature varied simultaneously every 5 days, for a total period of 56 days, using the same methodology as for the previous trials. In trial 3, the groups subjected to salinity and temperature oscillations were tested against groups subjected to fix rearing conditions (temperature: 25ºC; salinity: 30 ppt), which was named FIXED treatment. During trials 1, 2 and 3, the experimental diets (CONTROL and SEAWEED) were randomly assigned to each tank and fish were hand fed, twice a day, to apparent satiation and feed intake was recorded for each tank.

Table 2: Control and Supplemented Seaweed diets composition and Proximate composition.

	Dietary treatments	
	CONTROL	SEAWEED
Fish ingredients (% DM)		
Fishmeal Standard	10.0	10.0
Fishmeal SOLOR	20.0	20.0
Soy protein concentrate (Soycomil)	11.8	10.3
Wheat gluten	4.0	4.0
Corn gluten	8.0	8.0
Soybean meal 48	12.0	12.0
Rapeseed meal	5.0	5.0
Wheat meal	9.0	3.0
Peas gelatinized (Aquatex 8071)	3.2	3.2
Fish oil - COPPENS	6.5	6.5
Soybean oil	4.0	4.0
Rapeseed oil	4.0	4.0
Vit & Min Premix PV01	1.0	1.0
Binder (Kieselghur)	0.5	0.5
Antioxidant powder (Paramega)	0.2	0.2
MCP	0.5	0.5
L-Lysine	0.2	0.2
DL-Methionine	0.1	0.1
Gracilaria sp.	-	2.5
Ulva sp.	-	2.5
Fucus sp.	-	2.5
Proximate composition (%DM)		
Dry matter	94.7	94.8
Ash	8.6	10.6
Crude protein	47.8	47.9
Crude fat	19.1	19.3
Gross energy (kJ. g^{-1} DM)	22.7	22.4

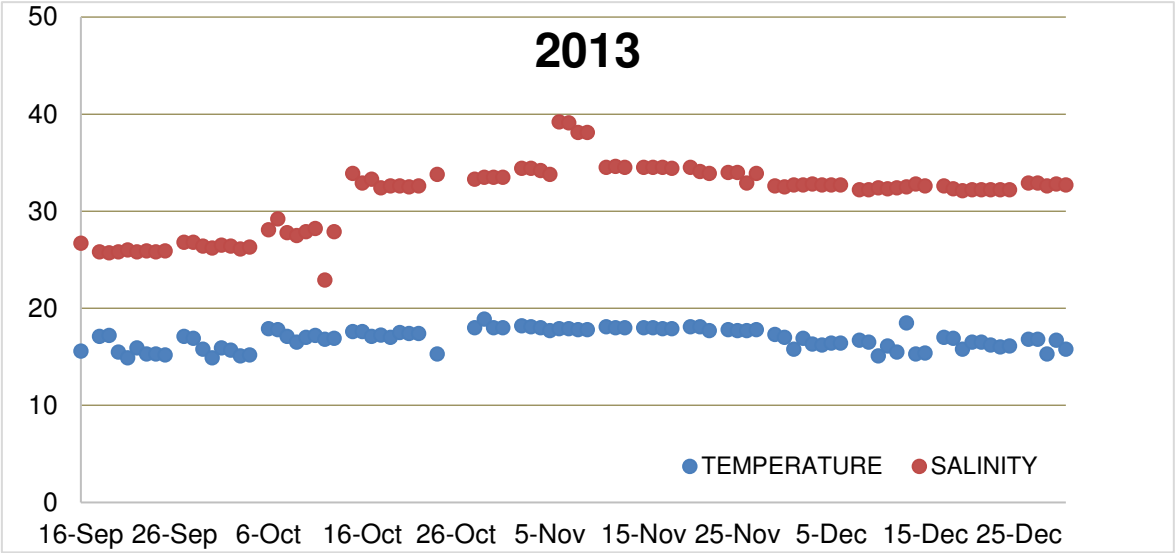

Figure 3: Salinity (ppt) and temperature (ºC) variation in Materaqua Lda. fish farm from September 16th to December 25th, year of 2013.

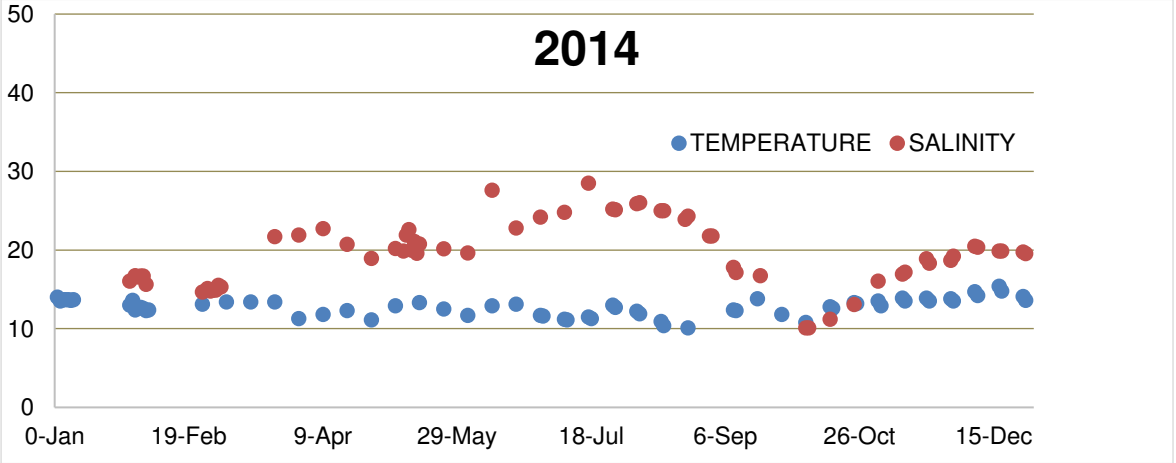

Figure 4: Salinity (ppt) and temperature (ºC) variation in Materaqua Lda. fish farm over the year of 2014.

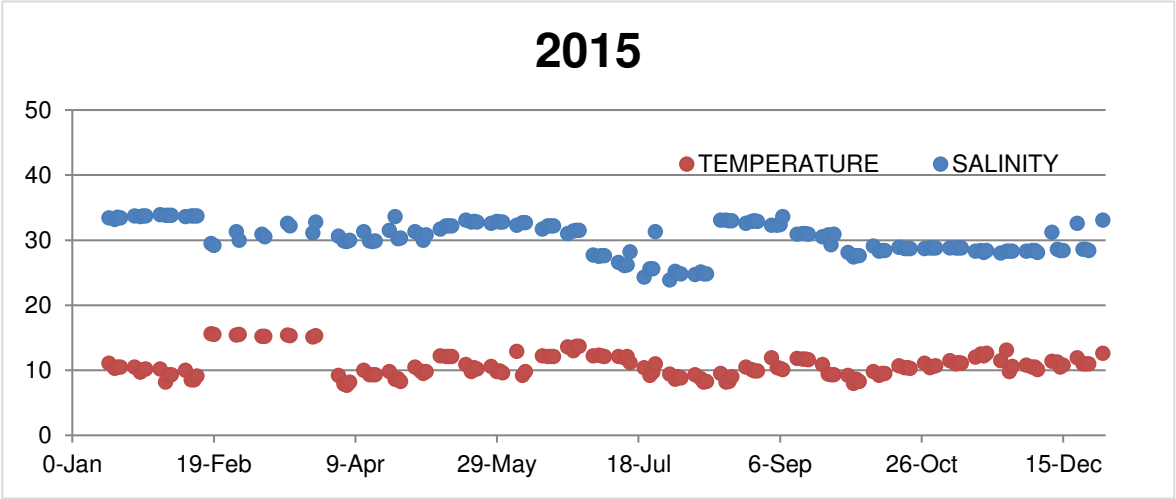

Figure 5: Salinity (ppt) and temperature (ºC) variation Materaqua Lda. fish farm over the year of 2015.

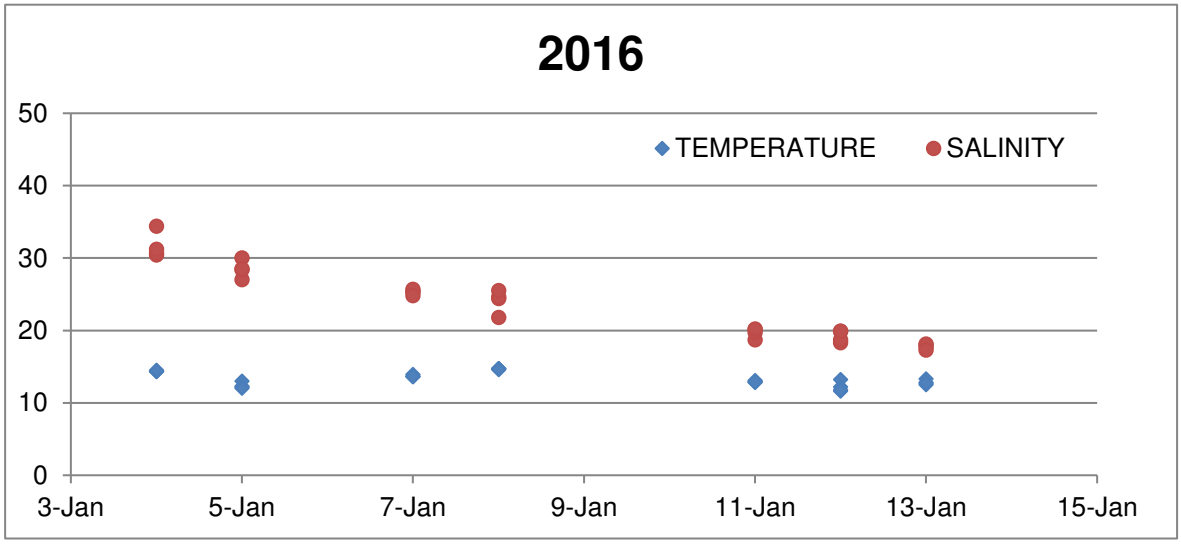

Figure 6: Salinity (ppt) and temperature (ºC) variation in Materaqua Lda. fish farm over the year of 2016, from January 1st to 13th.

Table 3: Water salinity (ppt, trial 1), temperature (ºC, trial 2) the combination of both (trial 3) for a period of 9 weeks (data are presented as mean ± standard deviation).

Week	Trial 1	Trial 2	Trial 3	
	Salinity	Temperature	Salinity	Temperature
1	13.5 ± 0.6	19.9 ± 0.8	39.8 ± 1.2	23.6 ± 1.5
2	17.3 ± 0.5	18.2 ± 0.1	43.2 ± 3.6	24.9 ± 0.5
3	15.1 ± 0.5	19.5 ± 0.5	39.8 ± 2.4	21.4 ± 3.9
4	30.4 ± 0.5	22.6 ± 1.1	35.8 ± 2.9	22.3 ± 0.8
5	13.1 ± 1.0	21.46 ± 0.6	34.6 ± 2.5	19.4 ± 1.4
6	18.3 ± 0.5	17.3 ± 1.4	39.2 ± 4.5	23.5 ± 2.3
7	25.5 ± 0.7	16.6 ± 0.7	38.8 ± 3.0	18.9 ± 1.7
8	24.4 ± 0.3	22.1 ± 2.1	30.1 ± 2.8	18.7 ± 0.4
9	25.7 ± 0.8	16.85 ± 3.7	38.4 ± 6.2	25.0 ± 1.2

In trial 3, the groups subjected to salinity and temperature oscillations were tested against groups subjected to a fix rearing conditions (temperature: 25 ºC; salinity: 30 ppt)

FISH AND EXPERIMENTAL FACILITIES

European seabass (*Dicentrarchus labrax)* juveniles were provided by IPMA, Olhão, Portugal and were kept in a quarantine system for 2 weeks at ICBAS-UP. After that, 234 fish (IBW of 10.5 ± 0.2 g) were distributed into 18 tanks with 13 fish per tank, and acclimatized to the rearing conditions for 15 days, at a temperature of 22 ºC. During that period, fish were fed *ad libitum* the CONTROL diet (non-supplemented). The experimental system was made of 18 tanks of 80 L each, connected to a single recirculation system (TMC® System 5000P Marine), 4 L min^{-1} per tank flow, with continuous aeration (Fig. 8).

Figure 7: Aquatic engineering Laboratory (ICBAS-UP).

Water parameters were monitored daily. During the salinity trial, oxygen levels were (7.8 mg.L^{-1}), pH (6.5-7.5), ammonia (<1mg / L), nitrite (<2mg.L^{-1}), and temperature 20 ºC. Salinity was measured 2 times a day for every tank. For trial 2 (temperature), fish were distributed into 18 tanks with a density of 11 fish per tank (initial body weight: 21.7 ± 1.19 g). Acclimatization lasted for 2 weeks, at a temperature of 19 ºC. In the trial 3 (Fig. 8), where temperature and salinity oscillated, fish were distributed into 11 tanks, with a density of 10 fish per tank (initial body weight: 41.77 ± 2.76 g). Seven tanks were subjected to temperature and salinity oscillations. In parallel, 4 other tanks were kept under constant conditions (20ºC temperature and 25 ppt salinity), used as a FIXED control group. Oxygen (8.7 mg.L^{-1}), pH (7), ammonia (<1mg / L) and nitrite (<2 mg / L) were daily monitored. For every trial, photoperiod was set for 12:12 h light:dark, and light sterilization of the rearing water was provided by 8 ultraviolet lamps of 54 Watts each.

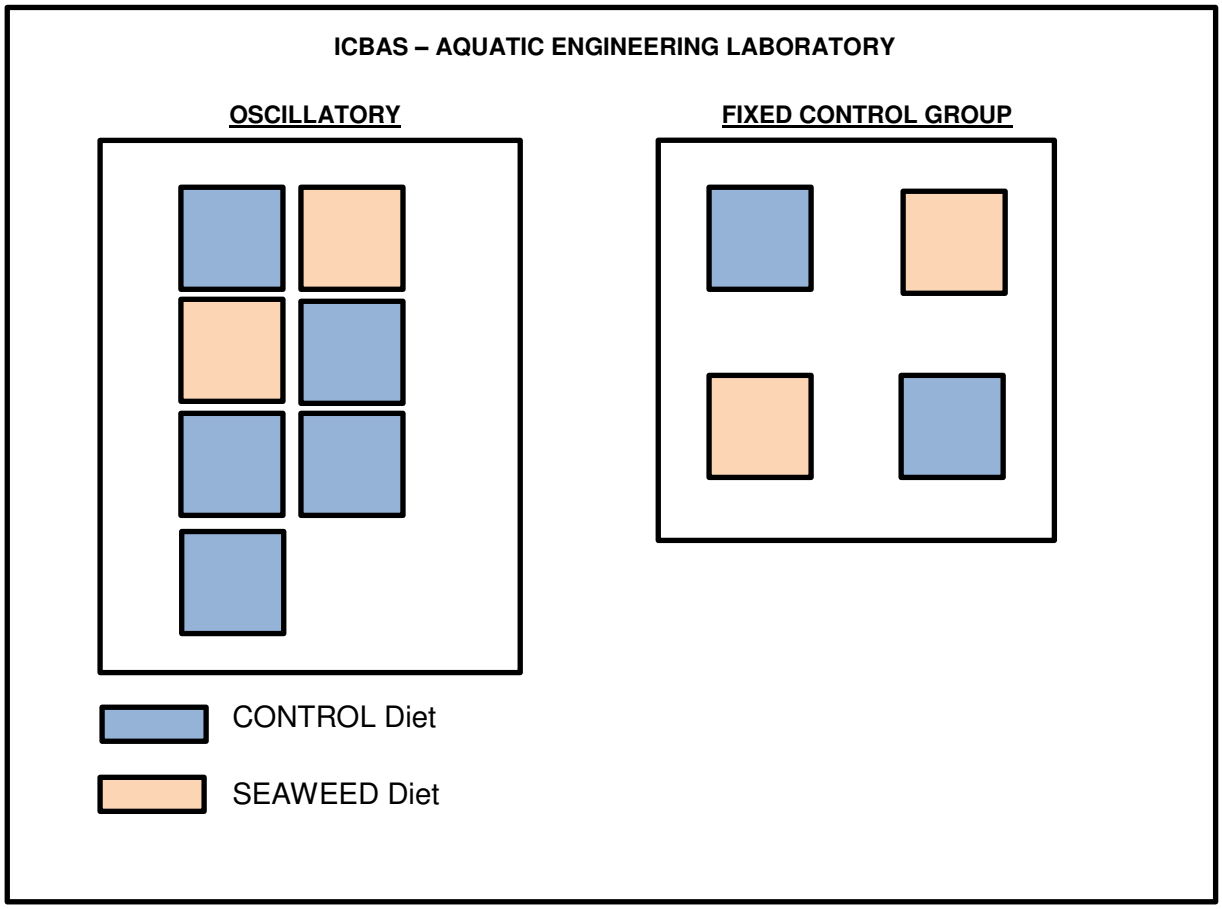

Figure 8: Schematic design of trial 3 located in ICBAS - Aquatic Engineering Laboratory. Each colored square represents an experimental tank.

SAMPLING

At the end of each trial (1, 2 and 3), sampling was performed. Two fish per tank were collected on the 1st trial, one in the 2nd and all the fish in the 3rd, and then anesthetized with ethylene glycol monobutyl ether (0.25 $mL.L^{-1}$). Weight and length were recorded of all fish from each tank to calculate growth performance parameters. Blood and liver were sampled from 2 fish/treatment and stored at -80°C until further analyzes. Both tissues were used for the analyses of immune (plasma) and oxidative stress (liver) parameters.

GROWTH PARAMETERS

Growth performance was determined using the following parameters: Daily growth index (DGI) = 100 x $[(FBW)^{1/3} - (IBW)^{1/3}]$ x trial duration in days, whereas FBW and IBW are the final and initial average body weights (g); Feed conversion ratio (FCR) = feed intake (g)/weight gain (g), whereas feed intake is (g)/fish; Protein efficiency ratio (PER) = weight gain (g)/protein intake (g).

HUMORAL IMMUNE PARAMETERS

The detailed procedures regarding the following analyses can be observed at the attachments.

Alternative complement pathway (ACH50)

The ACH50 was determined according to Sunyer *et al.* (1995). The main principle of this procedure is to evaluate the required plasma sample to cause 50% hemolysis in the added rabbit red blood cells. The alternative complement pathway units were defined as the concentration of serum giving 50% hemolysis of rabbit's blood cells. All analyses were conducted in triplicate.

Peroxidase

The total peroxidase content present in the plasma was measured according to Quade and Roth (1997). Using TMB (3.3′.5.5′-tetramethylbenzidine) as substrate, peroxidase activity was determined by hydrogen peroxide oxidation. A change of 1 optic density unit was assumed as 1 unit/ml of plasmatic peroxidase activity. Final unit is presented as enzymatic units.

Lysozyme

Lysozyme concentration was determined by the turbidimetric assay as described by Ellis (1990), measuring lysozyme action over *Micrococus lysodeikticusa* bacteria.

OXIDATIVE STRESS: ENZYMATIC AND NON-ENZYMATIC ANALYSES

The detailed procedures regarding the following analyses can be accessed at the attachments.

Livers were homogenized with a K-phosphate buffer (pH 7.4, 0.1 M) in a 1:15 (p / v) ratio. For the lipid peroxidation analysis, 2.5 µL of BHT 4% (2.6-Di-tert-butyl-4-methylphenol in methanol) were added to 150 µl of liver homogenate, for preservation, and stored at -80 ºC until quantification. For the remaining enzymes (CAT, GPX, GR and TG), samples were prepared by centrifuging liver homogenate (10 000 *G*, 20 min, 4 ºC) and the supernatant stored at -80 ºC. For CAT and GST quantification, the soluble protein content was required to be close to 0.7 $mg.mL^{-1}$. For this calibration, previous protein quantification and adequate dilutions were performed.

Protein quantification (mg. ml^{-1} homogenate)

Protein quantification was performed for calibration (CAT and GST) and final activity unit reference in all antioxidant enzymes. This quantification followed the Comassie binding principle of Bradford (1976).

Lipid peroxidation (nmoles MDA.mg $protein^{-1}$)

Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS essay using thiobarbituric acid as a reagent (Ohkawa *et al.*, 1979). TBARS essay measures malondialdehyde (MDA) present in the sample, which represents the main product of lipid peroxidation.

Catalase (µmol.min^{-1}.mg^{-1} protein)

With the soluble protein levels near 0.7 $mg.ml^{-1}$, activity quantification followed the principle of catalase action on peroxide hydrogen, as described by Claiborne (1985).

Glutathione s-transferase (pmol.min^{-1}.mg^{-1} protein)

This analysis was performed according to Habig *et al.* (1974). In this, CDNB (1-chloro-2,4-dinitrobenzene) is conjugated with GSH, forming the measurable conjugate of GSH-CDNB (1-chloro-2, 4-dinitrobenzene).

Glutathione peroxidase (µmol.min^{-1}.mg^{-1}.protein^{-1})

Glutathione peroxidase was quantified following the method by Mohandas *et al.* (1984). The reaction was measured by the formation of oxidized NADPH (NADP+), and presented in the final unit of NADP+.

Glutathione reductase (µmol.min^{-1}.mg.prot^{-1})

Glutathione reductase measurement assay was based on the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) to reduced glutathione (GSH) generated from an excess of oxidized glutathione (GSSG), as described by Cribb *et al.* (1989). This activity was quantified by the formation of oxidized NADPH (NADP+).

Total Glutathione (µmol.min^{-1}.mg.prot^{-1})

Total glutathione is quantified by reaction of GSH with DTNB (5.5'-dithio-bis-2-nitrobenzoic acid), which produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) quantified at 412 nm, as described by Baker *et al.* (1990). During the reaction, the GSSG is concomitantly reduced to GSH, hence enabling the measure of all glutathione.

STATISTICAL ANALYSES

Data was checked for normality (Grubbs' test) and homogeneity of variances (Levene´s test). Two-way (salinity and temperature trial) and one-way ANOVA (salinity trial and temperature trial) analyses were carried out using IBM SPSS statistics 20 - Windows XP/Vista/7/8 software package. Data transformation was applied when normality of the samples was not achieved. When data lacked homogeneity, Kruskal Wallis test was used. Tukey was used for pairwise comparisons between treatments. Confidence level of 95% was considered in all statistical analysis.

Results

TRIAL 1: SALINITY OSCILLATION

Growth performance

At the end of Trial 1, no mortality was registered. Fish grew from an initial mean body weight of 10.5 ± 0.7 g to a final mean body weight of 19.6 ± 1.3 g, in the 63-day period. No significant statistical differences were observed in the growth performance parameters (P≥0.05), except for feed intake and final body weight. Fish fed the SEAWEED diet had significantly lower (P<0.05) feed intake and final body weight (**Table 4**) than fish fed the CONTROL diet.

Table 4: Growth performance, feed utilization, and feed intake of seabass fed the experimental diets at the end of Trial 1.

Treatment	CONTROL	SEAWEED
Initial body weight (g)	10.6 ± 0.7	10.3 ± 0.9
Final body weight (g)	18.7 ± 3.8^{a}	16.5 ± 0.9^{b}
Weight gain (g)	8.10 ± 0.83	6.17 ± 1.07
Feed intake (g fish^{-1})	1.60 ± 0.93^{a}	1.50 ± 0.77^{b}
VFI (%ABW.day^{-1})	1.56 ± 0.08	1.61 ± 0.07
Protein intake (g)	0.72 ± 0.42	0.68 ± 0.35
FCR	1.38 ± 0.14	1.71 ± 0.15
DGI	0.94 ± 0.06	0.75 ± 0.09
PER	1.38 ± 0.11	1.33 ± 0.12

Values presented as mean (n = 6 tanks) ± SD. Different letters in the same row, for either CONTROL or SEAWEED diets, indicate significant differences (P<0.05; Final body weight P=0.048; Feed intake P=0.038). FCR, Feed conversion ratio = feed intake/weight gain; DGI, Daily growth index = 100 x [(Final body weight)$^{1/3}$ – (Initial body weight)$^{1/3}$]/days; PER, Protein efficiency ratio = weight gain/crude protein intake; ABW, Average body weight

Humoral immune parameters

Lysozyme and peroxidase activities

Dietary seaweed supplementation did not affect the plasma innate immune parameters, lysozyme (**Fig. 9**) and peroxidase (**Fig. 10**) activities (P≥0.05).

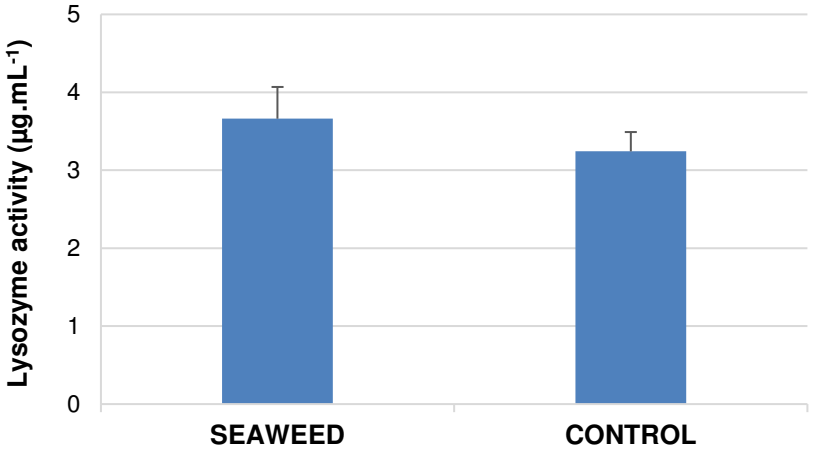

Figure 9: Plasma lysozyme activity (µg.mL^{-1}) in seabass fed the experimental diets at the end of Trial 1. Values are presented as mean ± standard deviation. Absence of letters indicates no significant differences (P≥0.05).

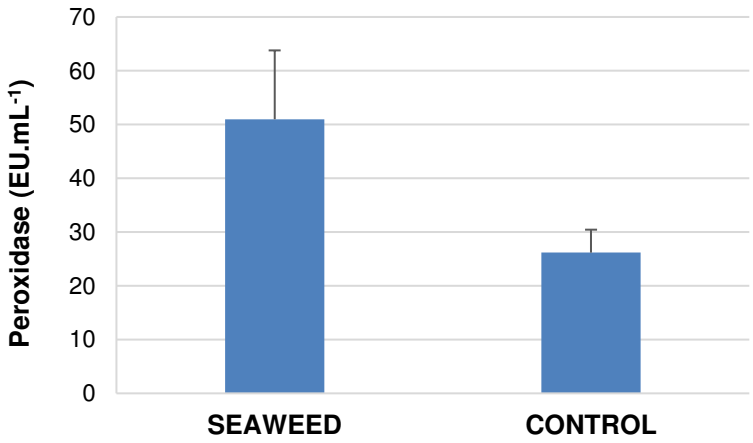

Figure 10: Plasma peroxidase activity (EU.mL^{-1}) in seabass fed the experimental diets at the end of Trial 1. Values are presented as mean ± standard deviation. Absence of letters indicates no significant differences (P≥0.05).

Oxidative stress: Enzymatic and non-enzymatic analyses

The indicators of enzymatic (CAT, GPx, GST) and non-enzymatic (TG, GSSG, GSH) antioxidant defenses and peroxidative damage (LPO) are presented in **Table 5**. At the end of Trial 1, CAT, GPx, and GST activities did not vary significantly (P≥0.05) in seabass fed the different dietary treatments. Similarly, no significant differences were observed on the LPO, TG, GSSG, GSH, and the GSH / GSSG.

Table 5: Enzymatic (CAT, GPx, GST) and non-enzymatic (LPO, TG, GSSG, GSH) bio-indicators analyzed in liver of seabass fed the experimental diets.

	CONTROL	**SEAWEED**
Catalase (µmol.min^{-1}.mg prot^{-1})	70.8 ± 10.5	73.0 ± 12.0
GPx (nmol.min^{-1}.mg prot^{-1})	0.14 ± 0.03	0.14 ± 0.02
GST (pmol.min^{-1}.mg prot^{-1})	17.2 ± 3.7	17.2 ± 4.5
LPO (nmol TBA.g^{-1})	19.07 ± 5.84	23.7 ± 8.9
TG (nmol.min^{-1}.mg prot^{-1})	0.14 ± 0.02	0.14 ± 0.03
GSSG (µmol min^{-1} mg^{-1})	0,061 ± 0,036	0,063 ± 0,036
GSH (µmol min^{-1} mg^{-1})	0,075 ± 0,036	0,077 ± 0,040
GSH-GSSG ratio	0,535 ± 0,226	0,634 ± 0,175

Catalase (CAT, µmol.min^{-1}.mg prot^{-1}), glutathione peroxidase (GPx, nmol.min^{-1}.mg prot^{-1}), glutathione s-transferase (GST, pmol.min^{-1}.mg prot^{-1}) Lipid Peroxidation (LPO, nmol TBA.g^{-1}), Total Glutathione (TG, nmol.min-1.mg prot-1), oxidized glutathione (GSSG, µmol min^{-1} mg^{-1}), reduced glutathione (GSH, µmol min^{-1} mg^{-1}) and the GSH-GSSG ratio. Values are presented as mean ± standard deviation. Absence of letters indicates no significant differences (P≥0.05).

TRIAL 2: TEMPERATURE

Growth performance

Fish grew from the initial mean body weight of 21.96 ± 1.09 g to a final mean body weight of 41.78 ± 3.26 g in the 63-day period. Weight gain (17-19 g), VFI (1.41-1.61 % ABW.day^{-1}), FCR (1.50-1.75), DGI (0.97-1.02 (% BW.day^{-}1) and PER (1.29-1.49) did not differ between the CONTROL and SEAWEED treatments (P≥0.05; **Table 6**)

Table 6: Growth performance parameters of seabass fed the experimental diets for 63 days.

Treatment	CONTROL	SEAWEED
Initial Weight (g)	22.4 ± 1.1	20.9 ± 1.3
Final Weight (g)	41.6 ± 2.4	38.1 ± 2.7
Weight Gain (g)	19.2 ± 1.4	17.3 ± 1.5
Feed intake (g fish^{-1})	28.6 ± 2.2	30.0 ± 3.3
VFI (%BW.day^{-1})	1.41 ± 0.09	1.61 ± 0.2
Protein intake (g)	12.9 ± 1.0	13.5 ± 1.5
FCR	1.50 ± 0.66	1.75 ± 0.26
DGI	1.02 ± 0.05	0.97 ± 0.04
PER	1.49 ± 0.06	1.29 ± 0.17

Values presented as mean (n = 6 tanks per treatment) ± SD. Absence of letters indicates no significant differences (ANOVA, P≥0.05). FCR, Feed conversion ratio = feed intake/weight gain; DGI, Daily growth index = 100 x [(Final body weight)$^{1/3}$ – (Initial body weight)$^{1/3}$]/days; PER, Protein efficiency ratio = weight gain/crude protein intake; ABW, Average body weight

Humoral immune parameters

Lysozyme and peroxidase activities

Lysozyme and peroxidase activities are presented in **Fig. 11** and **Fig. 12**, respectively. At the end of Trial 2, fish fed the SEAWEED diet had higher lysozyme activity than the CONTROL diet (P<0.05) while peroxidase activity was unaffected by dietary treatment (P≥0.05).

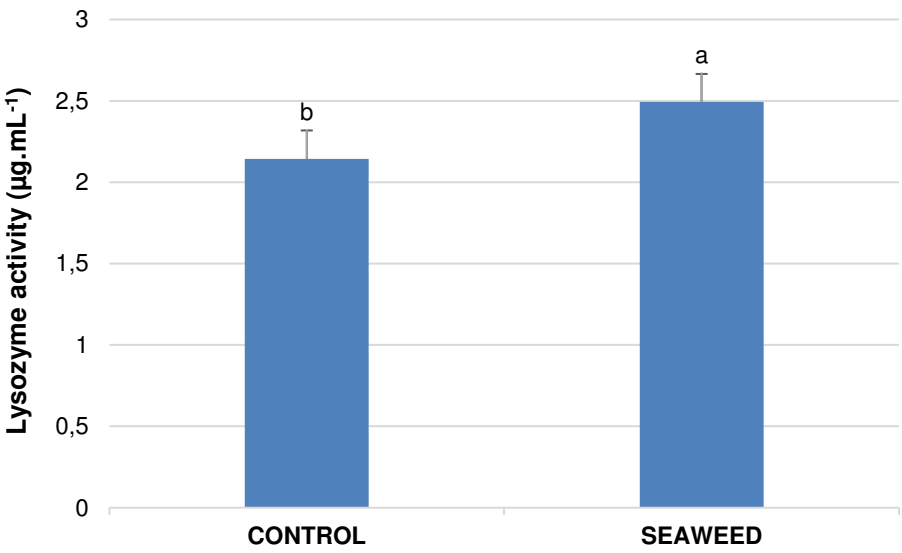

Figure 11: Lysozyme activity (µg.mL^{-1}) determined in the plasma of seabass fed the experimental diets at the end of Trial 2. Values are presented as mean ± SD. Different letters indicate significant differences (P<0.05).

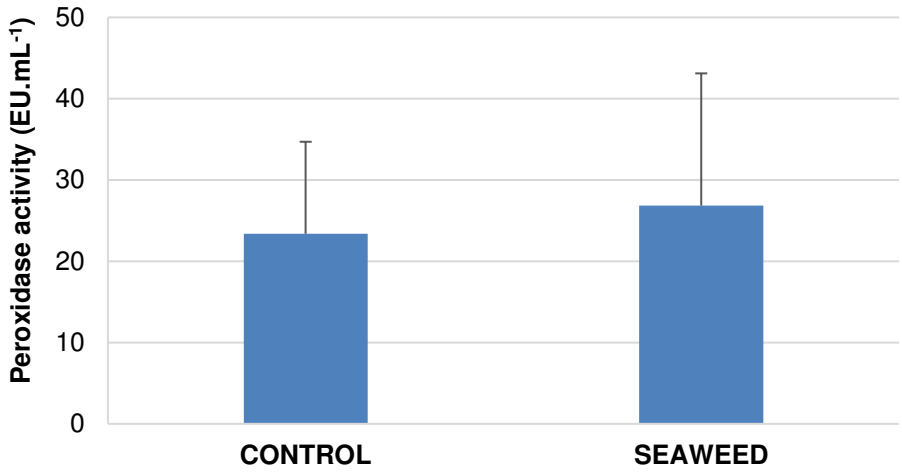

Figure 12: Peroxidase activity (EU.mL^{-1}) determined in the plasma of seabass fed the experimental diets at the end of Trial 2. Values are presented as mean ± SD. Absence of letters indicates no statistical differences (P≥0.05).

Oxidative stress: Enzymatic and non-enzymatic analyses

The indicators of enzymatic (CAT, GPx, GST) and non-enzymatic (TG, GSSG, GSH) antioxidant defenses and peroxidative damage (LPO) are presented in **Table 7**. At the end of Trial 1, CAT, GPx, and GST activities did not vary significantly (P≥0.05) in seabass fed the different dietary treatments. Similarly, no significant differences were observed on the LPO, TG, GSSG, GSH, and the GSH / GSSG.

Table 7 Enzymatic (CAT, GPx, GST), non-enzymatic (TG, GSSG, GSH) and peroxidative damage (LPO) bio-indicators in seabass fed the experimental diets during Trial 2.

	CONTROL	**SEAWEED**
Catalase (nmol.min^{-1}.mg protein^{-1})	46.3 ± 3.90	49.2 ± 9.17
GPx (nmol.min^{-1}.mg protein^{-1})	0.04 ± 0.01	0.04 ± 0.01
GST (pmol.min^{-1}.mg protein^{-1})	14.6 ± 3.5	14.5 ± 4.7
LPO (nmol TBA.g^{-1})	16.74 ± 2.78	20.19 ± 2.82
TG (nmol.min^{-1}.mg prot^{-1})	0.04 ± 0.011	0.04 ± 0.010
GSSG (µmol min^{-1} mg^{-1})	0,036 ± 0,012	0,034 ± 0,006
GSH (µmol min^{-1} mg^{-1})	0,01 ± 0,003	0,007 ± 0,003
GSH-GSSG ratio	0,35 ± 0,24	0,24 ± 0,10

Values are presented as mean ± SD. Absence of letters indicates no statistical differences (P≥0.05).

TRIAL 3: SALINITY AND TEMPERATURE

Growth performance

Growth performance parameters calculated at the end of Trial 3 are presented in Table 9. Fish grew from the initial mean body weight of 41.77 ± 2.76 g to a final mean body weight of 74.93 ± 9 g in the 63-day trial. There were no significant differences among the different experimental groups, SEAWEED and CONTROL, and also no differences between OSCILLATORY and FIXED groups, except for DGI. DGI was significantly higher in fish subjected to fixed temperature and salinity than in fish subjected to temperature and salinity oscillation (Kruskal Wallis, $P<0.05$).

Table 8: Growth performance parameters of seabass fed the experimental diets subjected to fixed or oscillatory conditions (Trial 3).

	OSCILLATORY		FIXED	
Treatment	**CONTROL**	**SEAWEED**	**CONTROL**	**SEAWEED**
Final body weight (g)	68.9 ± 4.0	74.2 ± 18.2	85.7 ± 7.3	80.1 ± 5.7
Weight gain (g)	26.4 ± 2.3	35.9 ± 15.9	42.8 ± 5.7	37.7 ± 6.1
Feed intake (g fish^{-1})	54.9 ± 6.3	38.7 ± 34.8	63.1 ± 8.3	59.5 ± 1.9
VFI (%ABW.day^{-1})	1.57 ± 0.12	1.63 ± 0.07	1.56 ± 0.10	1.54 ± 0.12
Protein intake (g)	24.7 ± 2.8	26.1 ± 5.9	28.4 ± 3.7	26.8 ± 0.9
FCR	2.08 ± 0.16	1.70 ± 0.38	1.47 ± 0.00	1.60 ± 0.31
DGI	0.97 ± 0.08^{b}	1.30 ± 0.43^{b}	1.44 ± 0.13^{a}	1.31 ± 0.18^{a}
PER	1.07 ± 0.08	1.34 ± 0.30	1.51 ± 0.00	1.41 ± 0.27

Values presented as mean (N = 6 tanks) ± SD. Absence of letters indicates no significant differences ($P\geq0.05$). FCR, Feed conversion ratio = feed intake/weight gain; DGI, Daily growth index = 100 x [(Final body weight)$^{1/3}$ – (Initial body weight)$^{1/3}$]/days; PER, Protein efficiency ratio = weight gain/crude protein intake; ABW, Average body weight

Humoral immune parameters

Lysozyme and peroxidase activities

At the end of Trial 3 no differences were observed in lysozyme activity (**Fig. 13**), regardless of the dietary and rearing conditions. Peroxidase activity did not vary between the dietary treatments, but was higher in fish subjected to FIXED rearing temperature and salinity (**Fig. 14**).

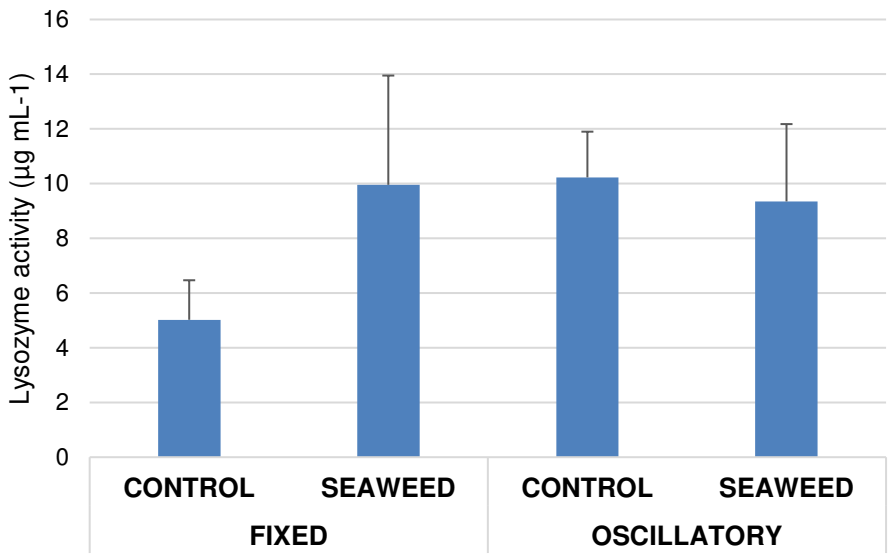

Figure 13: Lysozyme activity (µg mL^{-1}) determined in the liver of seabass fed the experimental diets subjected to fixed or oscillatory conditions (Trial 3) (mean ± SD). Absence of letters indicates no significant differences (P≥0.05).

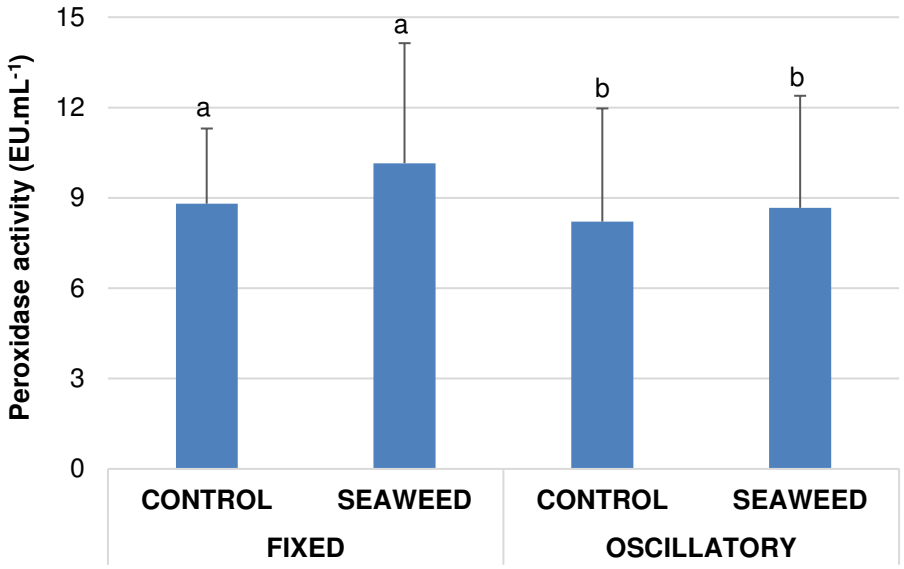

Figure 14: Peroxidase activity (EU.mL^{-1}) determined in the liver of seabass fed the experimental diets subjected to fixed or oscillation conditions (Trial 3). Values are presented as mean ± SD. Different letters indicate significant differences (P<0.05).

Oxidative stress: Enzymatic and non-enzymatic analyses

Catalase, glutathione peroxidase and glutathione s-transferase activities

The antioxidant enzymes (catalase, glutathione peroxidase, and glutathione s-transferase) did not vary with the different dietary or rearing (salinity and temperature) conditions (Table 10).

Table 9: Catalase, glutathione peroxidase (GPx), and glutathione s-transferase (GST) activities measured in the liver of seabass fed the experimental diets subjected to fixed or oscillation conditions (Trial 3).

	FIXED		OSCILLATORY	
	CONTROL	**SEAWEED**	**CONTROL**	**SEAWEED**
Catalase (nmol.min^{-1}.mg prot^{-1})	31,4 ± 17,8	42,4 ± 15,4	30,9 ± 11,1	25,6 ± 12,4
GPx (nmol.min^{-1}.mg prot^{-1})	0,51 ± 0,26	0,41 ± 0,17	0,62 ± 0,26	0,30 ± 0,19
GST (pmol.min^{-1}.mg prot^{-1})	558,2 ± 134,6	670,9 ± 232,7	542,1 ± 133,6	456,0 ± 68,4

Values are presented as mean (n=6) ± SD. Absence of letters indicates no significant differences (P≥0.05)

Lipid peroxidation

Lipid peroxidation was not affected by the dietary treatments nor by the changes in rearing temperature and salinity (P≥0.05; Fig. 15).

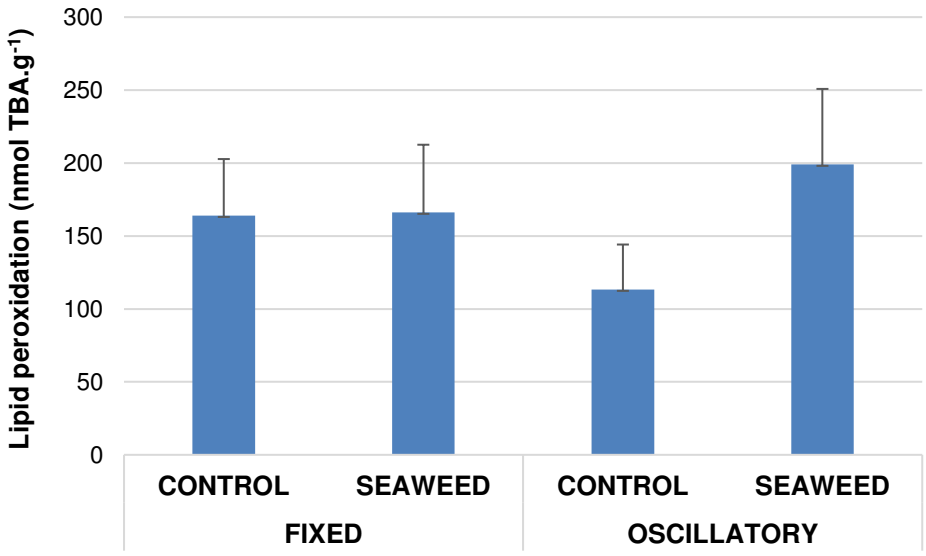

Figure 15: Lipid peroxidation (nmol TBA.g^{-1}) determined in the liver of seabass fed the experimental diets subjected to fixed or oscillatory conditions (Trial 3). Values are presented as mean ± SD. Absence of letters indicates no significant differences (P≥0.05).

Total Glutathione

Total glutathione concentration (Fig. 16) was not affected by the experimental diets; however, it was significantly higher (P<0.05) in seabass subjected to temperature and salinity changes (OSCILLATORY group), when compared to fish subjected to fixed conditions (FIXED group).

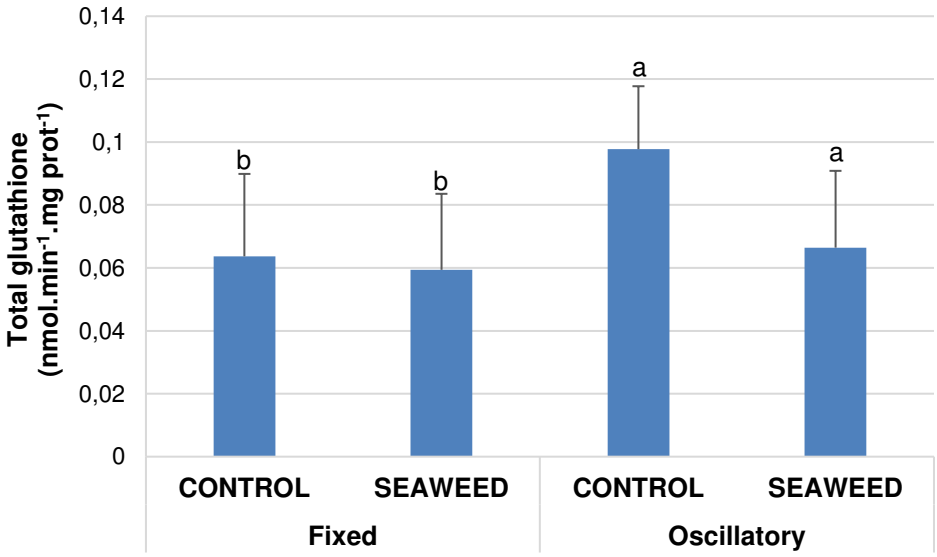

Figure 16: Total glutathione (nmol.min^{-1}.mg prot^{-1}) measured in the liver of seabass fed the experimental diets subjected to fixed or oscillation conditions (Trial 3). Values are presented as mean ± SD. Different letters indicate significant differences (P<0.05).

GSSG – oxidized glutathione and GSH – reduced glutathione

GSH activity was not affected by dietary treatment nor groups (P≥0.05) but GSSG was significant higher in the OSCILLATORY groups, independently of the diet (P<0.05; **Table 11)**.

Table 10: Oxidized glutathione (GSSG) and reduced glutathione (GSH) measured in liver of seabass fed the experimental diets subjected to fixed or oscillation conditions (Trial 3).

	FIXED		OSCILLATORY	
	CONTROL	**SEAWEED**	**CONTROL**	**SEAWEED**
GSSG (µmol min^{-1} mg^{-1})	0,016 ± 0,013^{b}	0,019 ± 0,013^{b}	0,065 ± 0,006^{a}	0,077 ± 0,015^{a}
GSH (µmol min^{-1} mg^{-1})	0,048 ± 0,027	0,041 ± 0,027	0,058 ± 0,032	0,049 ± 0,040
GSH/GSSG Ratio	2,720 ± 1,973	2,677 ± 3,231	0,906 ± 0,521	0,337 ± 0,111

Values are presented as means (n=6) ± SD. Different letters indicate significant differences (P<0.05).

Discussion

The economic importance of seabass for the European aquaculture sector compels this species to intensive rearing (Cabral and Costa, 2001; Vinagre *et al.*, 2009). In order to mitigate diseases outbreaks in intensive aquaculture, which cause substantial economic losses, it is necessary to develop disease control strategies based on a better understanding of the effects of husbandry methods and environmental stressors of farmed fish (Bowden *et al.*, 2007).

It is important not to rely solely on the use of antibiotics, which are undergoing strict regulations to limit their over-use, in order to prevent antibiotic resistant bacteria and minimize consumer's health risk (Cook *et al.*, 2003). Seaweed have been previously studied as potential immunostimulants in seabass (Peixoto *et al.*, 2016), as well as in tilapia (Güroy *et al.*, 2007), rainbow trout (Güroy *et al.*, 2011, 2013) among other fish species (Mustafa and Nakagawa, 1995; Güroy *et al.*, 2011; Peixoto *et al.*, 2016).

Growth Performance

In Trial 1, seaweed supplementation caused a decrease in growth performance and feed consumption of seabass subjected to salinity variations. Similar results were also observed by Davies *et al.* (1997), that reported a lower feed consumption in mullet (*Chelon labrosus*) feeding high levels of red algae. On the contrary, other published studies reported that the inclusion of seaweeds, such as *U. rigida* and *G. cornea* up to 10% and 5% respectively, did not cause adverse effects on growth performance and feed utilization in European seabass (Valente *et al.*, 2006). Also, Hashim and Saat (1992) observed an enhanced feeding activity and greater feed consumption of snakehead (*Channa striatus*) fed 5% *Ulva spp.* meal diet, compared to other seaweeds, indicating the possibility of the presence of a food attractant for this fish. Discrepancy in results using seaweed supplementation has been previously reported. Indeed, in the current salinity trial (Trial 1), the seaweeds used did not seem to positively affect the palatability of the diet for a carnivorous fish like seabass, since feed consumption was higher in the control group. The reduced final body weight could be related to the reduced feed consumption observed or to the presence of some anti-nutritional factors (ANF). Seaweeds contain polysaccharides like xylans, agar and alginates, which can limit their digestibility (Horie *et al.*, 1995), causing a lower growth performance and feed utilization, especially in carnivorous fish species. Other authors assume that the existence of ANF in seaweed diets can reduce its nutritional quality, interfere with the efficiency of digestive processes and reduce growth rates (Dallaire *et al.*,

2007; de Oliveira *et al*., 2009). Sáez *et al.* (2012) reported in *Sparus aurata* juveniles that *Ulva* meal contains anti-nutritional compounds that inhibit digestive proteases. In addition, de Oliveira *et al.* (2009) demonstrated the presence of many ANFs in *Gracilaria* species, which was also present in our diet. This may explain the lower weight gain observed in seabass fed the seaweed supplemented diet at such incorporation level.

In the temperature oscillatory trial (Trial 2), growth performance was not affected by the different diets. Similar results were obtained by other authors with the same level of seaweed supplementation in seabass (Bagni *et al*., 2000; Peixoto *et al*., 2016). Bagni *et al.* (2000) proposed that the lack of growth enhancement in seabass was an effect of the water temperature, which was below the optimal for the species.

According to Conides and Glamuzina (2006), the main parameter that affects the growth of seabass is the temperature/salinity combined effect. Similar to the results observed in the previously discussed trial, growth performance of seabass subjected to both salinity and temperature oscillations was not affected by dietary treatment. Kissil *et al.* (1992) also reported no differences in growth performance of grow-out gilthead seabream, after the inclusion of *Ulva* meal. However, regardless of the dietary treatment, the salinity and temperature oscillations (Trial 3) negatively affected growth performance (DGI), when compared to the FIXED group. Since temperature was fixed at 25 ºC in the FIXED group, these results are to be expected, as the temperature for optimal growth performance in seabass is between 25 and 28ºC (Conides and Glamuzina, 2006). Moreover, it would be expected to observe a lower weight gain in the OSCILLATORY group, but it was not the case in Trial 3. Although there is a tendency to lower values in final weight and weight gain, the high standard deviations difficult the interpretation of the results.

Peroxidase and Lysozyme

Lysozyme and peroxidase system are key components of fish immune defenses since they act against pathogens by directly disrupting their cell walls or thought the production of harmful chemicals, such as oxidative radicals (Nayak, 2010). Studies in fish immunity showed that seaweed compounds can modulate fish immunological response, playing a role in disease resistance in several species (Satoh, 1987; Dalmo and Seljelid, 1995; Castro *et al*., 2004). Lysozyme is a fundamental enzyme involved in the non-specific immune response of many fish species (Tort *et al*., 2004). However, lysozyme levels can vary considerably between fish species and, in most cases, it is positively correlated with disease resistance (Fevolden *et al*., 1994). In this study, when seabass was exposed to

salinity oscillations alone, the innate immune system response did not appear to be affected by seaweed supplementation, since lysozyme and peroxidase activities were not different between dietary groups. These results were also observed by Valente *et al.* (2016) in Nile tilapia, when fed a diet supplement with *Ulva* spp with 5% and 10% dietary inclusion. On the other hand, when temperature oscillated, lysozyme activity was significantly higher in fish fed the seaweed diet. These results are in agreement with Peixoto *et al.* (2016), which used similar seaweed supplementation in seabass, and with Bagni *et al.* (2000), which tested an immunostimulant diet in seabass. Increased peroxidase levels have been detected in rainbow trout fed diets with 5% *Gracilaria* sp. supplementation (Araújo *et al.*, 2016), while no differences were observed in Nile tilapia when fed diets supplemented with *Ulva* sp. at 5% and 10% levels (Valente *et al.*, 2016). In the current study, when temperature and salinity oscillated (Trial 3), peroxidase activity varied by changes of rearing temperature and salinity, but was unresponsive to the dietary supplementation.

Oxidative Stress

External factors such as rearing temperature and salinity are long-known to influence the oxidative stress indicators (Vinagre *et al.*, 2012). Antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), and levels of lipid peroxidation (LPO) are good indicators of ROS overproduction and induced oxidative damage in cells (Livingstone, 2001; Limón-Pacheco and Gonsebatt, 2009). Both for the salinity trial (Trial 1) and temperature trial (Trial 2), oxidative stress was not affected by dietary supplementation, which is in accordance with Peixoto *et al.* (2016).

Roche and Bogé (1996) tested the effect of temperature on oxidative stress biomarkers in seabass, concluding that lipid peroxidation and catalase activity were increased by thermal stress. In our study, catalase activity was not affected by dietary treatment or groups but results cannot be directly comparable with our study since the mentioned study was conducted 12 h after exposure. Kopecka and Pempkowiak (2008) also attributed the seasonal variation of catalase activity in European flounder (*Platichthys flesus*) to temperature oscillations (Kopecka and Pempkowiak, 2008).

Total Glutathione (TG) and oxidized glutathione (GSSG) were only affected in Trial 3, and only by temperature and salinity oscillation (Trial 3). TG showed higher activity in the OSCILLATORY group, mostly due to the GSSG fraction, since GSH was unaffected by any of the experimental treatment. Hight glutathione levels are related to improved antioxidant defenses (Sitjà-Bobadilla *et al.*, 2005). When comparing the TG with the LPO results, it

seems plausible to infer that the greater TG levels may have caused a decrease in the lipid damage (Sitjà-Bobadilla *et al*., 2005). Temperature influence in both TG and GSSG levels has already been reported in other studies, although these where evaluating seasonal variations in *Dicentrarchus labrax.* GSH/GSSG ratio is considered an index of the cellular redox status and a biomarker of oxidative damage (Sitjà-Bobadilla *et al*., 2005). According to Vinagre *et al.* (2012), lipid peroxidation and catalase activities in juvenile seabass are very sensitive to environmental temperature, particularly at temperatures outside the species optimal range. This contradicts this study results, in which FIXED and OSCILLATORY groups had no significantly differences in LPO and CAT.

Conclusion

For the salinity trial (Trial 1), the lower consumption and lower weight gain of fish fed supplemented diets indicate that either the quantity or combination of the seaweeds used as dietary supplement was not an appropriate supplementation for European seabass. No differences were observed in humoral immune parameters and oxidative stress.

When subjected to temperature oscillations, higher lysozyme activity was observed with the dietary seaweed supplementation.

When fish were subjected to salinity and temperature oscillation, seaweed supplementation did not affect any of the analyzed parameters.

The effects of the seaweed supplementation were not as pronounced as the abiotic factors (temperature and salinity). Further work should be carried out to test different seaweed species combination at different levels. In addition, the evaluation of processed seaweed (heat-treated seaweed) is of particular interest, since the thermal treatment of seaweed may inactivate several of the ANF that might have enshroud the potential positive results.

References

Abreu, M.H., Pereira, R., Yarish, C., Buschmann, A.H., Sousa-Pinto, I., 2011. IMTA with *Gracilaria vermiculophylla*: productivity and nutrient removal performance of the seaweed in a land-based pilot scale system. Aquaculture. 312, 77-87.

Aisa, Y., Miyakawa, Y., Nakazato, T., Shibata, H., Saito, K., Ikeda, Y., Kizaki, M., 2005. Fucoidan induces apoptosis of human HS-Sultan cells accompanied by activation of caspase-3 and down-regulation of ERK Pathways. American journal of hematology. 78, 7-14.

Ali, E.H., Hashem, M., Al-Salahy, M.B., 2011. Pathogenicity and oxidative stress in Nile tilapia caused by *Aphanomyces laevis* and *Phoma herbarum* isolated from farmed fish. Diseases of aquatic organisms. 94, 17-28.

Ameur, W.B., de Lapuente, J., El Megdiche, Y., Barhoumi, B., Trabelsi, S., Camps, L., Serret, J., Ramos-López, D., Gonzalez-Linares, J., Driss, M.R., 2012. Oxidative stress, genotoxicity and histopathology biomarker responses in mullet (Mugil cephalus) and sea bass (Dicentrarchus labrax) liver from Bizerte Lagoon (Tunisia). Marine pollution bulletin. 64, 241-251.

Angeles Esteban, M., Cuesta, A., Rodríguez, A., Meseguer, J., 2006. Effect of photoperiod on the fish innate immune system: a link between fish pineal gland and the immune system. Journal of pineal research. 41, 261-266.

Aoki, T., 1992. Chemotherapy and drug resistance in fish farms in Japan. Diseases in Asian aquaculture. 1, 519-529.

Araújo, M., Rema, P., Sousa-Pinto, I., Cunha, L.M., Peixoto, M.J., Pires, M.A., Seixas, F., Brotas, V., Beltrán, C., Valente, L.M., 2016. Dietary inclusion of IMTA-cultivated Gracilaria vermiculophylla in rainbow trout (Oncorhynchus mykiss) diets: effects on growth, intestinal morphology, tissue pigmentation, and immunological response. Journal of Applied Phycology. 28, 679-689.

Arunachalam, S., Reddy, S.R., 1979. Food intake, growth, food conversion, and body composition of catfish exposed to different salinities. Aquaculture. 16, 163-171.

Baba, T., Watase, Y., Yoshinaga, Y., 1993. Activation of mononuclear phagocyte function by levamisole immersion in carp. Bulletin of the Japanese Society of Scientific Fisheries (Japan).

Bagni, M., Archetti, L., Amadori, M., Marino, G., 2000. Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (Dicentrarchus labrax). Journal of Veterinary Medicine, Series B. 47, 745-751.

Bagni, M., Civitareale, C., Priori, A., Ballerini, A., Finoia, M., Brambilla, G., Marino, G., 2007. Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (Dicentrarchus labrax) and sea bream (Sparus aurata). Aquaculture. 263, 52-60.

Bajpai, S., Sharma, A., Gupta, M.N., 2005. Removal and recovery of antinutritional factors from soybean flour. Food chemistry. 89, 497-501.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical biochemistry. 190, 360-365.

Barnabé, G., 1990. Rearing bass and gilthead bream. Aquaculture. 2, 647-686.

Beitinger, T.L., Bennett, W.A., McCauley, R.W., 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. Environmental biology of fishes. 58, 237-275.

Berteau, O., Mulloy, B., 2003. Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. Glycobiology. 13, 29R-40R.

Björnsson, B., Ólafsdóttir, S.R., 2006. Effects of water quality and stocking density on growth performance of juvenile cod (Gadus morhua L.). ICES Journal of Marine Science: Journal du Conseil. 63, 326-334.

Blaber, S., 1997. Fish and fisheries in tropical estuaries. Springer Science & Business Media.
Boeuf, G., Payan, P., 2001. How should salinity influence fish growth? Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 130, 411-423.
Boshra, H., Li, J., Sunyer, J., 2006. Recent advances on the complement system of teleost fish. Fish & shellfish immunology. 20, 239-262.
Bowden, T.J., Thompson, K.D., Morgan, A.L., Gratacap, R.M., Nikoskelainen, S., 2007. Seasonal variation and the immune response: a fish perspective. Fish & shellfish immunology. 22, 695-706.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 72, 248-254.
Bragadóttir, M., 2001. Endogenous antioxidants in fish. University of Iceland.
Briand, X., Morand, P., 1997. Anaerobic digestion of Ulva sp. 1. Relationship between Ulva composition and methanisation. Journal of Applied Phycology. 9, 511-524.
Bricknell, I., Dalmo, R.A., 2005. The use of immunostimulants in fish larval aquaculture. Fish Shellfish Immunol. 19, 457-472.
Burrells, C., Williams, P., Forno, P., 2001. Dietary nucleotides: a novel supplement in fish feeds: 1. Effects on resistance to disease in salmonids. Aquaculture. 199, 159-169.
Buschmann, A.H., Troell, M., Kautsky, N., 2001. Integrated algal farming: a review. Cahiers de Biologie marine. 42, 83-90.
Cabral, H., Costa, M.J., 2001. Abundance, feeding ecology and growth of 0-group sea bass, Dicentrarchus labrax, within the nursery areas of the Tagus estuary. Journal of the Marine Biological Association of the UK. 81, 679-682.
Castro, R., Zarra, I., Lamas, J., 2004. Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes. Aquaculture. 229, 67-78.
Cecchini, S., Saroglia, M., 2002. Antibody response in sea bass (Dicentrarchus labrax L.) in relation to water temperature and oxygenation. Aquaculture Research. 33, 607-613.
Chen, D., Ainsworth, A., 1992. Glucan administration potentiates immune defence mechanisms of channel catfish, Ictalurus punctatus Rafinesque. Journal of Fish Diseases. 15, 295-304.
Claiborne, A., 1985. Catalase activity. CRC handbook of methods for oxygen radical research. 1, 283-284.
Conides, A., Parpoura, A., Fotis, G., 1997. Study on the effects of salinity on the fry of the euryhaline species gilthead sea bream (Sparus aurata L. 1758). Journal of Aquaculture in the Tropics. 12, 297-304.
Conides, A.J., Glamuzina, B., 2006. Laboratory simulation of the effects of environmental salinity on acclimation, feeding and growth of wild-caught juveniles of European sea bass Dicentrarchus labrax and gilthead sea bream, Sparus aurata. Aquaculture. 256, 235-245.
Cook, M.T., Hayball, P.J., Hutchinson, W., Nowak, B.F., Hayball, J.D., 2003. Administration of a commercial immunostimulant preparation, EcoActiva™ as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (Pagrus auratus, Sparidae (Bloch and Schneider)) in winter. Fish & Shellfish Immunology. 14, 333-345.
Cornish-Bowden, A., 1979. Chapter 2 - Introduction to enzyme kinetics, Fundamentals of Enzyme Kinetics. Butterworth-Heinemann, pp. 16-38.
Costas, B., Aragão, C., Mancera, J.M., Dinis, M.T., Conceição, L.E., 2008. High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole Solea senegalensis (Kaup 1858) juveniles. Aquaculture Research. 39, 1-9.
Cox, S., Abu-Ghannam, N., Gupta, S., 2010. An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds.

Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of a microplate reader in an assay of glutathione reductase using 5, 5′-dithiobis (2-nitrobenzoic acid). Analytical biochemistry. 183, 195-196.
Dalla Via, J., Villani, P., Gasteiger, E., Niederstätter, H., 1998. Oxygen consumption in sea bass fingerling Dicentrarchus labrax exposed to acute salinity and temperature changes: metabolic basis for maximum stocking density estimations. Aquaculture. 169, 303-313.
Dallaire, V., Lessard, P., Vandenberg, G., de la Noüe, J., 2007. Effect of algal incorporation on growth, survival and carcass composition of rainbow trout (Oncorhynchus mykiss) fry. Bioresource technology. 98, 1433-1439.
Dalmo, R., Seljelid, R., 1995. The immunomodulatory effect of LPS, laminaran and sulphated laminaran [β (I, 3)-D-glucan] on Atlantic salmon, Salmo salar L., macrophages in vitro. Journal of fish diseases. 18, 175-185.
Dantagnan, P., Hernández, A., Borquez, A., Mansilla, A., 2009. Inclusion of macroalgae meal (Macrocystis pyrifera) as feed ingredient for rainbow trout (Oncorhynchus mykiss): effect on flesh fatty acid composition. Aquaculture Research. 41, 87-94.
Davies, S., Brown, M., Camilleri, M., 1997. Preliminary assessment of the seaweed Porphyra purpurea in artificial diets for thick-lipped grey mullet (Chelon labrosus). Aquaculture. 152, 249-258.
Day, J.W., 1989. Estuarine ecology. John Wiley & Sons.
de Oliveira, M.N., Freitas, A.L.P., Carvalho, A.F.U., Sampaio, T.M.T., Farias, D.F., Teixeira, D.I.A., Gouveia, S.T., Pereira, J.G., 2009. Nutritive and non-nutritive attributes of washed-up seaweeds from the coast of Ceará, Brazil. Food chemistry. 115, 254-259.
Dendrinos, P., Thorpe, J., 1985. Effects of reduced salinity on growth and body composition in the European bass Dicentrarchus labrax (L.). Aquaculture. 49, 333-358.
Díaz-Rosales, P., Burmeister, A., Aguilera, J., Korbee, N., Moriñigo, M., Figueroa, F., Chabrillón, M., Arijo, S., Lindequist, U., Balebona, M., 2005. Screening of algal extracts as potential stimulants of chemotaxis and respiratory burst activity of phagocytes from sole (Solea senegalensis). Bulletin of the European Association of Fish Pathologists. 25, 9-19.
Diler, I., Tekinay, A.A., Guroy, D., Guroy, B.K., Soyuturk, M., 2007. Effects of Ulva rigida on the growth, feed intake and body composition of common carp, Cyprinus carpio L.
Dominguez, M., Takemura, A., Tsuchiya, M., 2005. Effects of changes in environmental factors on the non-specific immune response of Nile tilapia, *Oreochromis niloticus* L. Aquaculture Research. 36, 391-397.
Dworjanyn, S.A., Pirozzi, I., Liu, W., 2007. The effect of the addition of algae feeding stimulants to artificial diets for the sea urchin Tripneustes gratilla. Aquaculture. 273, 624-633.
Ellis, A.E., 1990. Lysozyme assays. Techniques in fish immunology. 1, 101-103.
Ellis, T., North, B., Scott, A., Bromage, N., Porter, M., Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. Journal of Fish Biology. 61, 493-531.
Ergün, S., Soyutürk, M., Güroy, B., Güroy, D., Merrifield, D., 2009. Influence of Ulva meal on growth, feed utilization, and body composition of juvenile Nile tilapia (Oreochromis niloticus) at two levels of dietary lipid. Aquaculture International. 17, 355-361.
Eroglu, A., Dogan, Z., Kanak, E., Atli, G., Canli, M., 2015. Effects of heavy metals (Cd, Cu, Cr, Pb, Zn) on fish glutathione metabolism. Environmental Science and Pollution Research. 22, 3229-3237.
FAO, 2014. The State of World Fisheries and Aquaculture: Opportunities and challenges. in: NATIONS, F.A.A.O.O.T.U. (Ed.), Rome, 2014.
FAO, 2016. Aquaculture Department (2009) The state of world fisheries and aquaculture 2008. Food and agriculture organization of the United Nations, Rome.

FAO, F.a.A.D., 2012. The State of World Fisheries and Aquaculture. FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, Rome.
Fevolden, S., Røed, K., Gjerde, B., 1994. Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon; correlations to disease resistance. Fish & Shellfish Immunology. 4, 507-519.
Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature. 408, 239-247.
Fishbase, 2016. European seabass - Dicentrarchus labrax (Linnaeus, 1758), http://www.fishbase.org/summary/Dicentrarchus-labrax.html.
Francis, G., Makkar, H.P., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199, 197-227.
García-Casal, M.N., Pereira, A.C., Leets, I., Ramírez, J., Quiroga, M.F., 2007. High iron content and bioavailability in humans from four species of marine algae. The Journal of nutrition. 137, 2691-2695.
Guerriero, G., Di Finizio, A., Ciarcia, G., 2002. Stress-induced changes of plasma antioxidants in aquacultured sea bass, *Dicentrarchus labrax*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 132, 205-211.
Güroy, B., Ergün, S., Merrifield, D.L., Güroy, D., 2013. Effect of autoclaved Ulva meal on growth performance, nutrient utilization and fatty acid profile of rainbow trout, Oncorhynchus mykiss. Aquaculture International. 21, 605-615.
Güroy, B.K., CİRİK, Ş., Güroy, D., Sanver, F., TEKİNAY, A.A., 2007. Effects of Ulva rigida and Cystoseira barbata meals as a feed additive on growth performance, feed utilization, and body composition of Nile tilapia, Oreochromis niloticus. Turkish Journal of Veterinary and Animal Sciences. 31, 91-97.
Güroy, D., Güroy, B., Merrifield, D., Ergün, S., Tekinay, A., Yiğit, M., 2011. Effect of dietary Ulva and Spirulina on weight loss and body composition of rainbow trout, Oncorhynchus mykiss (Walbaum), during a starvation period. Journal of animal physiology and animal nutrition. 95, 320-327.
Gutt, J., 1985. The growth of juvenile flounders (Platichthys flesus L.) at salinities of 0, 5, 15 and 35%%. Journal of Applied Ichthyology. 1, 17-26.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. Journal of biological Chemistry. 249, 7130-7139.
Haroun-Bouhedja, F., Ellouali, M., Sinquin, C., Boisson-Vidal, C., 2000. Relationship between sulfate groups and biological activities of fucans. Thrombosis research. 100, 453-459.
Hashim, R., Saat, M.A.M., 1992. The utilization of seaweed meals as binding agents in pelleted feeds for snakehead (Channa striatus) fry and their effects on growth. Aquaculture. 108, 299-308.
Hemmingson, J., Falshaw, R., Furneaux, R., Thompson, K., 2006. Structure and antiviral activity of the galactofucan sulfates extracted from Undaria pinnatifida (Phaeophyta). Journal of Applied Phycology. 18, 185-193.
Heo, S.-J., Park, E.-J., Lee, K.-W., Jeon, Y.-J., 2005. Antioxidant activities of enzymatic extracts from brown seaweeds. Bioresource Technology. 96, 1613-1623.
Hoar, W.S., Randall, D.J., Iwama, G., Nakanishi, T., 1997. The fish immune system: organism, pathogen, and environment. Academic Press.
Horie, Y., Sugase, K., Horie, K., 1995. Physiological differences of soluble and insoluble dietary fibre fractions of brown algae and mushrooms in pepsin activity in vitro and protein digestibility. Asian Pacific Journal of Clinical Nutrition. 4, 251-255.
ICES, 2013. Report of the Working Group on Assessment of New MoU Species (WGNEW).
Imsland, A.K., Björnsson, B.T., Gunnarsson, S., Foss, A., Stefansson, S.O., 2007. Temperature and salinity effects on plasma insulin-like growth factor-I concentrations and growth in juvenile turbot (Scophthalmus maximus). Aquaculture. 271, 546-552.

INE, 2013. Instituto Nacional de Estatistica.
Jeney, G., Jeney, Z., 2002. Application of immunostimulants for modulation of the non-specific defense mechanisms in sturgeon hybrid: Acipenser ruthenus× A. baerii. Journal of Applied Ichthyology. 18, 416-419.
Jeney, G., Galeotti, M., Volpatti, D., 1994. Effect of immunostimulation on the non specific immune response of sea bass Dicentrarchus labrax, International Symposium on Aquatic Animal Health, Seattle, Washington, USA, 4e8 September.
Jiménez-Escrig, A., Gomez-Ordonez, E., Rupérez, P., 2011. Seaweed as a source of novel nutraceuticals: sulfated polysaccharides and peptides. Adv Food Nutr Res. 64, 325-337.
Jollès, P., Jollès, J., 1984. What's new in lysozyme research? Molecular and cellular biochemistry. 63, 165-189.
Jørgensen, J.B., Robertsen, B., 1995. Yeast β-glucan stimulates respiratory burst activity of Atlantic salmon (Salmo salar L.) macrophages. Developmental & Comparative Immunology. 19, 43-57.
Kajita, Y., Sakai, M., Atsuta, S., Kobayashi, M., 1990. The immunomodulatory effects of levamisole on rainbow trout, Oncorhynchus mykiss. 魚病研究. 25, 93-98.
Katzenback, B.A., Katakura, F., Belosevic, M., 2012. Regulation of teleost macrophage and neutrophil cell development by growth factors and transcription factors. INTECH Open Access Publisher.
Killie, J.-E.A., Jørgensen, T.Ø., 1994. Immunoregulation in fish I: Intramolecular-induced suppression of antibody responses to haptenated protein antigens studied in Atlantic salmon (Salmo salar L). Developmental & Comparative Immunology. 18, 123-136.
Kim, J.-H., Lee, J.-S., Kang, J.-C., 2012. Effect of inorganic mercury on hematological and antioxidant parameters on olive flounder Paralichthys olivaceus. Fisheries and aquatic sciences. 15, 215-220.
Kissil, G., Lupatsch, I., Neori, A., 1992. Approaches to fish feed in Israeli mariculture as a result of environmental constraints. Collected reprints. 18. 359Á369.
Kitao, T., Yoshida, T., Anderson, D., Dixon, O., Blanch, A., 1987. Immunostimulation of antibody-producing cells and humoral antibody to fish bacterins by a biological response modifier. Journal of Fish Biology. 31, 87-91.
Kopecka, J., Pempkowiak, J., 2008. Temporal and spatial variations of selected biomarker activities in flounder (Platichthys flesus) collected in the Baltic proper. Ecotoxicology and environmental safety. 70, 379-391.
Koumans-van Diepen, J.C.E., 1993. Characterisation of fish leucocytes. An immunocytochemical and functional study in carp (Cyprinus carpio L.), Department of Experimental Animal Morphology and Cell Biology. Wageningen Agricultural University, Netherlands.
Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S., Shimeno, H., 2003. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. Biochemical pharmacology. 65, 173-179.
Lahaye, M., Gomez-Pinchetti, J.L., del Rio, M.J., Garcia-Reina, G., 1995. Natural decoloration, composition and increase in dietary fibre content of an edible marine algae, Ulva rigida (Chlorophyta), grown under different nitrogen conditions. Journal of the Science of Food and Agriculture. 68, 99-104.
Lambert, Y., Dutil, J.-D., Munro, J., 1994. Effects of intermediate and low salinity conditions on growth rate and food conversion of Atlantic cod (Gadus morhua). Canadian Journal of Fisheries and Aquatic Sciences. 51, 1569-1576.
Lemarie, G., Dosdat, A., Covès, D., Dutto, G., Gasset, E., Person-Le Ruyet, J., 2004. Effect of chronic ammonia exposure on growth of European seabass (Dicentrarchus labrax) juveniles. Aquaculture. 229, 479-491.
Leonard, S., Sweeney, T., Bahar, B., Lynch, B., O'Doherty, J., 2011. Effects of dietary seaweed extract supplementation in sows and post-weaned pigs on performance, intestinal morphology, intestinal microflora and immune status. British journal of nutrition. 106, 688-699.

Liao, W.-R., Lin, J.-Y., Shieh, W.-Y., Jeng, W.-L., Huang, R., 2003. Antibiotic activity of lectins from marine algae against marine vibrios. Journal of Industrial Microbiology and Biotechnology. 30, 433-439.
Liener, I.E., 1994. Implications of antinutritional components in soybean foods. Critical Reviews in Food Science & Nutrition. 34, 31-67.
Limón-Pacheco, J., Gonsebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 674, 137-147.
Livingstone, D., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine pollution bulletin. 42, 656-666.
Livingstone, D., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue de Medecine Veterinaire. 154, 427-430.
Low, C., Wadsworth, S., Burrells, C., Secombes, C., 2003. Expression of immune genes in turbot (Scophthalmus maximus) fed a nucleotide-supplemented diet. Aquaculture. 221, 23-40.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology. 101, 13-30.
Lushchak, V.I., Bagnyukova, T.V., 2006. Effects of different environmental oxygen levels on free radical processes in fish. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 144, 283-289.
Magnuson, J.J., Crowder, L.B., Medvick, P.A., 1979. Temperature as an ecological resource. American Zoologist. 19, 331-343.
Makkar, H., 1993. Antinutritional factors in foods for livestock. BSAP occasional publication: An occasional publication of the British Society of Animal Production.
Makkar, H.P., Tran, G., Heuzé, V., Giger-Reverdin, S., Lessire, M., Lebas, F., Ankers, P., 2016. Seaweeds for livestock diets: A review. Animal Feed Science and Technology. 212, 1-17.
Marinho, G., Nunes, C., Sousa-Pinto, I., Pereira, R., Rema, P., Valente, L.M., 2013. The IMTA-cultivated Chlorophyta Ulva spp. as a sustainable ingredient in Nile tilapia (Oreochromis niloticus) diets. Journal of applied phycology. 25, 1359-1367.
Mata, L., Schuenhoff, A., Santos, R., 2010. A direct comparison of the performance of the seaweed biofilters, Asparagopsis armata and Ulva rigida. Journal of Applied Phycology. 22, 639-644.
Mohandas, J., Marshall, J.J., Duggin, G.G., Horvath, J.S., Tiller, D.J., 1984. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney: possible implications in analgesic nephropathy. Biochemical pharmacology. 33, 1801-1807.
Montero, D., Izquierdo, M., Tort, L., Robaina, L., Vergara, J., 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, Sparus aurata, juveniles. Fish Physiology and Biochemistry. 20, 53-60.
Montgomery, W.L., Gerking, S.D., 1980. Marine macroalgae as foods for fishes: an evaluation of potential food quality. Environmental Biology of Fishes. 5, 143-153.
Morgan, J.D., Iwama, G.K., 1991. Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow and steelhead trout (Oncorhynchus mykiss) and fall chinook salmon (Oncorhynchus tshawytscha). Canadian Journal of Fisheries and Aquatic Sciences. 48, 2083-2094.
Mosser, M., Hettler, W., 1989. Routine metabolism of juvenile spot, Leiostomus xanthurus. J Fish Biol. 35, 703-707.
Mustafa, M.G., Nakagawa, H., 1995. A review: dietary benefits of algae as an additive in fish feed. Israeli Journal of Aquaculture-Bamidgeh. 47, 155-162.
Nagler, P.L., Glenn, E.P., Nelson, S.G., Napolean, S., 2003. Effects of fertilization treatment and stocking density on the growth and production of the economic seaweed Gracilaria parvispora (Rhodophyta) in cage culture at Molokai, Hawaii. Aquaculture. 219, 379-391.

Nakagawa, H., 1997. Effect of dietary algae on improvement of lipid metabolism in fish. Biomedicine & pharmacotherapy. 51, 345-348.
Nakagawa, H., Umino, T., Tasaka, Y., 1997. Usefulness of Ascophyllum meal as a feed additive for red sea bream, Pagrus major. Aquaculture. 151, 275-281.
Nakajima, K., 1991. Effects of dimethyl-beta-propiothetin on growth and thrust power of rainbow trout. Bulletin of the Japanese Society of Scientific Fisheries (Japan).
Nakajima, K., Uchida, A., Ishida, Y., 1989. Effect of a feeding attractant, dimethyl-beta-propiothetin, on growth of marine fish [red sea bream, yellowtail and flounder]. Bulletin of the Japanese Society of Scientific Fisheries (Japan).
Nakatsuji, T., Gallo, R.L., 2012. Antimicrobial peptides: old molecules with new ideas. Journal of Investigative Dermatology. 132, 887-895.
Narasimhan, M.K., Pavithra, S.K., Krishnan, V., Chandrasekaran, M., 2013. In vitro analysis of antioxidant, antimicrobial and antiproliferative activity of Enteromorpha antenna, Enteromorpha linza and Gracilaria corticata extracts. Jundishapur Journal of Natural Pharmaceutical Products. 8, 151.
Nayak, S., 2010. Probiotics and immunity: a fish perspective. Fish & shellfish immunology. 29, 2-14.
Neori, A., Shpigel, M., Ben-Ezra, D., 2000. A sustainable integrated system for culture of fish, seaweed and abalone. Aquaculture. 186, 279-291.
Neyrinck, A.M., Mouson, A., Delzenne, N.M., 2007. Dietary supplementation with laminarin, a fermentable marine β (1–3) glucan, protects against hepatotoxicity induced by LPS in rat by modulating immune response in the hepatic tissue. International immunopharmacology. 7, 1497-1506.
Nyberg, C.D., Thomsen, M.S., Wallentinus, I., 2009. Flora and fauna associated with the introduced red alga Gracilaria vermiculophylla. European Journal of Phycology. 44, 395-403.
Obach, A., Quentel, C., Laurencin, F.B., 1993. Dicen trarch us la brax. Diseases of Aquatic Organisms. 15, 175-185.
Ogier de Baulny, M., Quentel, C., Fournier, V., Lamour, F., Le Gouvello, R., 1996. Effect of long-term oral administration of β-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot Scophthalmus maximus. Diseases of Aquatic Organisms. 26, 139-147.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry. 95, 351-358.
Olsen, R., Henderson, R., 1997. Muscle fatty acid composition and oxidative stress indices of Arctic charr, Salvelinus alpinus (L.), in relation to dietary polyunsaturated fatty acid levels and temperature. Aquaculture Nutrition. 3, 227-238.
Olsen, R.E., Sundell, K., Mayhew, T.M., Myklebust, R., Ringø, E., 2005. Acute stress alters intestinal function of rainbow trout, Oncorhynchus mykiss (Walbaum). Aquaculture. 250, 480-495.
Olsen, R.E., Sundell, K., Hansen, T., Hemre, G.-I., Myklebust, R., Mayhew, T.M., Ringø, E., 2002. Acute stress alters the intestinal lining of Atlantic salmon, Salmo salar L.: An electron microscopical study. Fish physiology and biochemistry. 26, 211-221.
Ortiz, J., Romero, N., Robert, P., Araya, J., Lopez-Hernández, J., Bozzo, C., Navarrete, E., Osorio, A., Rios, A., 2006. Dietary fiber, amino acid, fatty acid and tocopherol contents of the edible seaweeds Ulva lactuca and Durvillaea antarctica. Food chemistry. 99, 98-104.
Partridge, G.J., Jenkins, G.I., 2002. The effect of salinity on growth and survival of juvenile black bream (Acanthopagrus butcheri). Aquaculture. 210, 219-230.
Pascual, P., Pedrajas, J., Toribio, F., López-Barea, J., Peinado, J., 2003. Effect of food deprivation on oxidative stress biomarkers in fish (*Sparus aurata*). Chemico-biological interactions. 145, 191-199.
Peddie, S., Zou, J., Secombes, C.J., 2002. Immunostimulation in the rainbow trout (Oncorhynchus mykiss) following intraperitoneal administration of Ergosan. Veterinary immunology and immunopathology. 86, 101-113.

Peixoto, M.J., Salas-Leitón, E., Pereira, L.F., Queiroz, A., Magalhães, F., Pereira, R., Abreu, H., Reis, P.A., Gonçalves, J.F.M., de Almeida Ozório, R.O., 2016. Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (Dicentrarchus labrax). Aquaculture Reports. 3, 189-197.

Peng, C., Hong-BO, S., Di, X., Song, Q., 2009. Progress in Gracilaria biology and developmental utilization: main issues and prospective. Reviews in Fisheries Science. 17, 494-504.

Pereira, R., Kraemer, G., Yarish, C., Sousa-Pinto, I., 2008. Nitrogen uptake by gametophytes of Porphyra dioica (Bangiales, Rhodophyta) under controlled-culture conditions. European Journal of Phycology. 43, 107-118.

Pereira, R., Valente, L.M., Sousa-Pinto, I., Rema, P., 2012. Apparent nutrient digestibility of seaweeds by rainbow trout (Oncorhynchus mykiss) and Nile tilapia (Oreochromis niloticus). Algal Research. 1, 77-82.

Person-Le Ruyet, J., Mahe, K., Le Bayon, N., Le Delliou, H., 2004. Effects of temperature on growth and metabolism in a Mediterranean population of European sea bass, Dicentrarchus labrax. Aquaculture. 237, 269-280.

Person-Le Ruyet, J., Lacut, A., Le Bayon, N., Le Roux, A., Pichavant, K., Quéméner, L., 2003. Effects of repeated hypoxic shocks on growth and metabolism of turbot juveniles. Aquatic Living Resources. 16, 25-34.

Pham, M.A., Lee, K., Lee, B., Lim, S., Kim, S., Lee, Y., Heo, M., Lee, K., 2006. Effects of dietary Hizikia fusiformis on growth and immune responses in juvenile olive flounder (Paralichthys olivaceus). ASIAN AUSTRALASIAN JOURNAL OF ANIMAL SCIENCES. 19, 1769.

Pichavant, K., Person-Le-Ruyet, J., Bayon, N.L., Severe, A., Roux, A.L., Boeuf, G., 2001. Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. Journal of Fish Biology. 59, 875-883.

Pickering, A., 1992. Rainbow trout husbandry: management of the stress response. Aquaculture. 100, 125-139.

Pickering, A., Pottinger, T., 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. Fish physiology and biochemistry. 7, 253-258.

Pickett, G.D., Pawson, M.G., 1994. Sea Bass: Biology. Springer Science & Business Media.

Pinchetti, J.L.G., del Campo Fernández, E., Díez, P.M., Reina, G.G., 1998. Nitrogen availability influences the biochemical composition and photosynthesis of tank-cultivated Ulva rigida (Chlorophyta). Journal of Applied Phycology. 10, 383-389.

Plaza, M., Cifuentes, A., Ibáñez, E., 2008. In the search of new functional food ingredients from algae. Trends in Food Science & Technology. 19, 31-39.

Priede, I.G., 1985. Metabolic scope in fishes, Fish Energetics. Springer, pp. 33-64.

Quade, M.J., Roth, J.A., 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology and Immunopathology. 58, 239-248.

Reynolds, W.W., Casterlin, M.E., 1979. Behavioral thermoregulation and the “final preferendum” paradigm. American zoologist. 19, 211-224.

Roberts, R.J., 2012. Fish pathology. John Wiley & Sons, USA.

Roche, H., Bogé, G., 1996. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. Marine Environmental Research. 41, 27-43.

Sáez, M., Martinez, T., Alarcon, J., 2012. Effect of dietary inclusion of seaweeds on intestinal proteolytic activity of juvenile seabream, Sparus aurata, 15th Int. Symp. of Nutrition and Feeding of Fish P.

Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture. 172, 63-92.

Sakai, M., Otubo, T., Atsuta, S., Kobayashi, M., 1993. Enhancement of resistance to bacterial infection in rainbow trout, Oncorhynchus mykiss (Walbaum), by oral administration of bovine lactoferrin. Journal of Fish Diseases. 16, 239-247.

Sakai, M., Kamiya, H., Ishii, S., Atsuta, S., Kobayashi, M., 1992. The immunostimulating effects of chitin in rainbow trout, Oncorhynchus mykiss. Diseases in Asian aquaculture. 1, 413-417.

Sangiao-Alvarellos, S., Laiz-Carrión, R., Guzmán, J.M., del Río, M.P.M., Miguez, J.M., Mancera, J.M., Soengas, J.L., 2003. Acclimation of S. aurata to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 285, R897-R907.

Sangiao-Alvarellos, S., Guzmán, J.M., Láiz-Carrión, R., Míguez, J.M., Martín Del Río, M.P., Mancera, J.M., Soengas, J.L., 2005. Interactive effects of high stocking density and food deprivation on carbohydrate metabolism in several tissues of gilthead sea bream Sparus auratus. Journal of Experimental Zoology Part A: Comparative Experimental Biology. 303, 761-775.

Sarropoulou, E., Fernandes, J.M., Mitter, K., Magoulas, A., Mulero, V., Sepulcre, M.P., Figueras, A., Novoa, B., Kotoulas, G., 2010. Evolution of a multifunctional gene: the warm temperature acclimation protein Wap65 in the European seabass Dicentrarchus labrax. Molecular phylogenetics and evolution. 55, 640-649.

Satoh, K., 1987. Effect of Ulva meal supplementation on disease resistance of red sea bream. 日本水産学会誌. 53, 1115-1120.

Scapigliati, G., Romano, N., Buonocore, F., Picchietti, S., Baldassini, M., Prugnoli, D., Galice, A., Meloni, S., Secombes, C., Mazzini, M., 2002. The immune system of sea bass, Dicentrarchus labrax, reared in aquaculture. Developmental & Comparative Immunology. 26, 151-160.

Schijf, J., Ebling, A.M., 2010. Investigation of the Ionic Strength Dependence of Ulva lactuca Acid Functional Group p K as by Manual Alkalimetric Titrations. Environmental science & technology. 44, 1644-1649.

Schmidt-Nielsen, K., 1997. Animal physiology: adaptation and environment. Cambridge University Press.

Schuenhoff, A., Shpigel, M., Lupatsch, I., Ashkenazi, A., Msuya, F.E., Neori, A., 2003. A semi-recirculating, integrated system for the culture of fish and seaweed. Aquaculture. 221, 167-181.

Secombes, C., Hardie, L., Daniels, G., 1996. Cytokines in fish: an update. Fish & Shellfish Immunology. 6, 291-304.

Sengupta, P., Garrity, P., 2013. Sensing temperature. Current Biology. 23, R304-R307.

Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z., 2011. Metals as a cause of oxidative stress in fish: a review. Vet Med. 56, 537-546.

Shields, R.J., Lupatsch, I., 2012. Algae for aquaculture and animal feeds. J Anim Sci. 21, 23-37.

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (Sparus aurata). Aquaculture. 249, 387-400.

Soler-Vila, A., Coughlan, S., Guiry, M.D., Kraan, S., 2009. The red alga Porphyra dioica as a fish-feed ingredient for rainbow trout (Oncorhynchus mykiss): effects on growth, feed efficiency, and carcass composition. Journal of Applied Phycology. 21, 617-624.

Sunyer, J., Gomez, E., Tort, L., Navarro, V., Quesada, J., 1995. Physiological responses and depression of humoral components of the immune system in gilthead sea bream (Sparus aurata) following daily acute stress. Canadian Journal of Fisheries and Aquatic Sciences. 52, 2339-2346.

Thanigaivel, S., Chandrasekaran, N., Mukherjee, A., Thomas, J., 2015. Investigation of seaweed extracts as a source of treatment against bacterial fish pathogen. Aquaculture. 448, 82-86.

Thomsen, M., McGlathery, K.J., 2007. Stress tolerance of the invasive macroalgae Codium fragile and Gracilaria vermiculophylla in a soft-bottom turbid lagoon. Biological Invasions. 9, 499-513.

Torrecillas, S., Makol, A., Caballero, M., Montero, D., Robaina, L., Real, F., Sweetman, J., Tort, L., Izquierdo, M., 2007. Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. Fish & Shellfish Immunology. 23, 969-981.

Tort, L., Rotllant, J., Rovira, L., 1998. Immunological suppression in gilthead sea bream *Sparus aurata* of the North-West Mediterranean at low temperatures. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 120, 175-179.

Tort, L., Balasch, J., MacKenzie, S., 2004. Fish health challenge after stress. Indicators of immunocompetence. Contributions to Science. 2, 443-454.

Tsevis, N., Klaoudatos, S., Conides, A., 1992. Food conversion budget in sea bass, Dicentrarchus labrax, fingerlings under two different feeding frequency patterns. Aquaculture. 101, 293-304.

Vadstein, O., 1997. The use of immunostimulation in marine larviculture: possibilities and challenges. Aquaculture. 155, 401-417.

Valente, L., Gouveia, A., Rema, P., Matos, J., Gomes, E., Pinto, I., 2006. Evaluation of three seaweeds Gracilaria bursa-pastoris, Ulva rigida and Gracilaria cornea as dietary ingredients in European sea bass (Dicentrarchus labrax) juveniles. Aquaculture. 252, 85-91.

Valente, L.M., Araújo, M., Batista, S., Peixoto, M.J., Sousa-Pinto, I., Brotas, V., Cunha, L.M., Rema, P., 2016. Carotenoid deposition, flesh quality and immunological response of Nile tilapia fed increasing levels of IMTA-cultivated Ulva spp. Journal of Applied Phycology. 28, 691-701.

Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental toxicology and pharmacology. 13, 57-149.

Vinagre, C., Ferreira, T., Matos, L., Costa, M., Cabral, H., 2009. Latitudinal gradients in growth and spawning of sea bass, Dicentrarchus labrax, and their relationship with temperature and photoperiod. Estuarine, Coastal and Shelf Science. 81, 375-380.

Vinagre, C., Madeira, D., Narciso, L., Cabral, H.N., Diniz, M., 2012. Effect of temperature on oxidative stress in fish: lipid peroxidation and catalase activity in the muscle of juvenile seabass, Dicentrarchus labrax. Ecological indicators. 23, 274-279.

Waagbø, R., Glette, J., Sandnes, K., Hemre, G., 1994. Influence of dietary carbohydrate on blood chemistry, immunity and disease resistance in Atlantic salmon, Salmo salar L. Journal of Fish Diseases. 17, 245-258.

Wahbeh, M.I., 1997. Amino acid and fatty acid profiles of four species of macroalgae from Aqaba and their suitability for use in fish diets. Aquaculture. 159, 101-109.

Wassef, E., El Masry, M., Mikhail, F., 2001. Growth enhancement and muscle structure of striped mullet, Mugil cephalus L., fingerlings by feeding algal meal-based diets. Aquaculture Research. 32, 315-322.

Wassef, E.A., El-Sayed, A.-F.M., Sakr, E.M., 2013. Pterocladia (Rhodophyta) and Ulva (Chlorophyta) as feed supplements for European seabass, Dicentrarchus labrax L., fry. Journal of applied phycology. 25, 1369-1376.

Wassef, E.A., El-Sayed, A.F.M., Kandeel, K.M., Sakr, E.M., 2005. Evaluation of Pterocladia (Rhodophyta) and Ulva (Chlorophyta) meals as additives to gilthead seabream Sparus aurata diets. Egypt J Aquat Res. 31, 321-332.

Xu, B., Hirata, H., 1990. Effects of feed additive Ulva reproduced in feedback culture system on the growth and color of red sea bream, Pagrus major. Suisanzoshoku (Japan).

Yano, T., Mangindaan, Mangindaan, R.E., 1989. Enhancement of the resistance of carp Cyprinus carpio to experimental Edwardsiella tarda infection, by some. BETA.-1, 3-glucans. 日本水産学会誌. 55, 1815-1819.
Yi, Y.-H., Chang, Y.-J., 1994. Physiological effects of seamustard supplement diet on the growth and body composition of young rockfish, Sebastes schlegeli. Korean Journal of Fisheries and Aquatic Sciences. 27, 69-82.
Yokoyama, H., Ishihi, Y., 2010. Bioindicator and biofilter function of Ulva spp.(Chlorophyta) for dissolved inorganic nitrogen discharged from a coastal fish farm—potential role in integrated multi-trophic aquaculture. Aquaculture. 310, 74-83.
Yoshida, T., Sakai, M., Kitao, T., Khlil, S.M., Araki, S., Saitoh, R., Ineno, T., Inglis, V., 1993. Immunomodulatory effects of the fermented products of chicken egg, EF203, on rainbow trout, Oncorhynchus mykiss. Aquaculture. 109, 207-214.
Zanuy, S., Carrillo, M., 1984. La salinité: un moyen pour retarder la ponte du bar. L'aquaculture du bar. INRA Publication, Paris, 73-80.
Zong, W.-X., Thompson, C.B., 2006. Necrotic death as a cell fate. Genes & development. 20, 1-15.

Attachments

LABORATORY ANALYSIS PROCEDURES

Complement system – (Sunyer *et al.*, 1995)

First, the sample of rabbit red blood cells was cleaned with physiological serum (NaCl 0.9%) in a 1:4 ratio (blood:serum), and centrifuged for 5 min. at 2000 *G*. We collected a clear pink solution with 2.8x108 cell/ml (RaRBC), determined using a 1:200 dilution and resorting to cell counting and a microscope. We measured the correct dilution required for 50% hemolysis, by adding a previously calculated amount of rabbit's blood solution and 10µl of our plasma samples to the 96-well microplate. In this experiment, we tested serial dilutions from 1:8 to 1:96. Incubation occurred for 100 minutes, with continuous agitation, and stopped with a solution composed of 0.1 % gelatin, 5mM sodium barbiturate, 0.13 mM sodium chloride and 20mM EDTA, pH of 7.3.

Finally, microplates were centrifuged at 122*G* for 2.5 min. 150µl were then pipetted to a new microplate and the lysed cells measured at the absorbance of 414 nm wavelength. The alternative complement pathway (ACH50) units were defined as the concentration of plasma giving 50% hemolysis of RaRBC. All analyses were conducted in triplicates.

Peroxidase - (Quade and Roth, 1997)

To estimate the plasmatic peroxidase content, 135 µl of a stabilizing environment of Hank's Balanced Salt Solution (HBSS Ca^{2+} free) was added to a flat bottom 96-well microplate, followed by 15µl of plasma sample, 50µl of tetremethylbenzidine hydrochloride (TMB) and 50 µl of hydrogen peroxide (H_2O_2), allowing reaction to occur. After 2 min, 50 µl of 2 mM of sulfuric acid (H_2SO_4) to stop the reaction. The principle behind the reaction is that TMB can act as a hydrogen donor for the reduction of hydrogen peroxide to water by peroxidase enzymes, such as the exocytosis myeloperoxidase and eosinophilic peroxidase. By adding sulfuric acid, TMB turns yellow and the absorbance is read at 450 nm. Final unit is presented as enzymatic units, with one enzymatic being defined as the amount producing an absorbance change of 1.62 units.

Lysozyme - (Ellis, 1990)

This procedure starts with the preparation of a 0.5 mg/ml of *Micrococus lysodeikticusa* solution in a 0.05M sodium phosphate dibasic (Na_2HPO_4) buffer. This bacterial solution will be used as target subtract for the lysozyme present in the samples. 15 consecutive dilutions of Hen egg-white lysozyme (HEWL), from 1 mg/ml to 0.0049µg/ml, are used to find the

equation from where we can calculate the actual concentration. A reaction mix of 15µl plasma sample and 250µl of bacterial suspension was then read at 450nm wavelength in 2 timings, 30 seconds and 270 seconds.

Protein quantification - (Bradford, 1976)

Initially, a standard curve of 250 µl BioRad® with 10µl of a known concentration of bovine gamma globulin (0, 0.2, 0.5 and 1 $mg.ml^{-1}$) was created. After a 15 min incubation in the dark (to avoid light damage), and a final reading at 600 nm, we were able to calculate the concentration of our samples placed under the same conditions.

Lipid peroxidation - (Ohkawa *et al.*, 1979)

The reaction complex is composed of: 150 µl of liver homogenate, 500 µl of TBA (Thiobarbituric acid), 500 µl of TCA (Trichloroacetic acid), 400 µl of Tris-HCl (Trisaminometane) at 60 mM with 0.1 mM DTPA (diethylene triamine pentaacetic acid). After an incubation period of 60 min at 100 ºC, a centrifugation of 11500*G*, at 25 ºC, for 5 min was made. Samples were then read at 535nm. The principle of this analysis is that the MDA-TBA adducts formed by the reaction of MDA and TBA under high temperature (100°C) and acidic conditions (TCA) can be measured calorimetrically at 535 nm. With the absorbance of this measurement and the extinction coefficient of the MDA-TBA complex (1.56 x 105 $M^{-1}.cm^{-1}$), the actual concentration of the lipid, peroxidation product (MDA), can be quantified. Units are presented as nmoles of MDA formed.mg $protein^{-1}$.

Catalase - (Claiborne, 1985)

The reaction complex was based on 15 µl of sample, 135 µl of K-phosphate buffer at 0.05 M (pH 7) and 150 µl of peroxide hydrogen (H_2O_2) at 0.03 M. The peroxide hydrogen concentration was then accessed by spectrophotometry at 240 nm for the first minute. This absorbance, along with the peroxide hydrogen extinction coefficient (40 $M^{-1}.cm^{-1}$) allowed the calculation of the catalase activity slope. Final unit is presented $\mu mol.min^{-1}$.protein mg^{-1}.

Glutathione s-transferase - (Habig *et al.*, 1974)

For this reaction, 100 µl of sample was added to 4.95 ml of phosphate buffer (0.1M; pH 6.5), 900µL of reduced glutathione (GSH) at 10mM and to 150µL of CDNB (1-chloro-2,4-dinitrobenzene) at 10mM. Spectrophotometer readings were made at 340nm wavelength every 20 secs. for 5 min., after all reagents were combined. The enzyme Glutathione-S-transferase (GST) conjugates CDNB (substrate) with glutathione (GSH) originating a complex. The formation of this compound (ε =9.6 $mM^{-1}.cm^{-1}$) can be monitored at 340 nm,

registering an increase in the absorbance over time. Results are expressed in µmol GSH-CDNB conjugate formed in min^{-1}.protein mg^{-1}.

Glutathione peroxidase - (Mohandas *et al.*, 1984)

This requires the peptide GSH and peroxide hydrogen to recreate a reaction mix composed of: 840 µl of K-Phosphate 0.05M (pH 7.0) with EDTA 1mM and Sodium azide (1mM) and GR at 1 U/ml; 50 µL GSH 4mM, 50µL NADPH (Nicotinamide adenine dinucleotide phosphate) 0.8mM, 10 µL H2O2 0.5mM and finally 50 µL of sample. Measurements were recorded at 340nm for 5 min (every 20 seconds = 15 readings total). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is indicative of GPX activity, since GPX is the rate limiting factor of the coupled reactions.

Glutathione reductase - (Cribb *et al.*, 1989)

In this method, a reaction buffer (pH 7.0) with 0.0085g of NADPH, 0.0033g of GSSG and 0.0098g of DTPA is mixed. Then, 15µL of sample (or homogenization buffer as blank) plus 285 µL of reaction buffer is added to a microplate. Readings were made at 340 nm wave length for 1 min (every 20 sec readings = 3 total readings). The absorbance increasing rate is directly proportional to the amount of glutathione reductase in the sample.

Total Glutathione - (Baker *et al.*, 1990)

Total glutathione (TG) required a previous treatment, where 10 µl of ultra-pure water were added to 200µl sample and left to incubate for 1 hour at 25 ºC. After this, 200 µl of 12% pure TCA (Trichloroacetic acid) were added, incubated for 1 hour at 4ºC, followed by a centrifugation at 10000*G* for 5 min. The reaction *per se* included 20µL of treated sample, 175µL Na-K phosphate (0.2M pH 8.0), 30µL of NADPH, 60µL of DTNB and 15µL of GR. Samples were read at 412nm wavelength for 10 min.

POSTER PRESENTATION

IJUP – Meeting of young researchers at University of Porto
February 17th 2016 – Poster Presentation

Effect of dietary seaweed supplementation on growth performance in seabass (*Dicentrarchus labrax*) subjected to rearing salinity oscillations.

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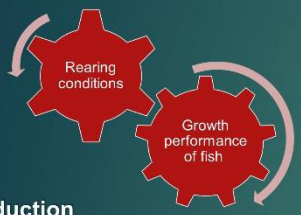


Introduction

Fish suffer changes of ion transport mechanisms induced by the different environmental salinities, which may cause an increase in oxygen consumption. As a consequence, energy budget is channeled for covering osmoregulation energy expenditure, instead for growth. In fact, salinity seems to modify multiple aspects related to growth, including standard metabolic rate, feed intake and feed conversion efficiency. Nutritional studies have shown that seaweed supplementation may improve growth performance and dietary energy utilization, when fish are reared under suboptimal conditions.

Aim: Evaluate the effect of dietary seaweed supplementation on growth performance in European seabass (*Dicentrarchus labrax*) subjected to salinity oscillations.

Material and methods

- **Trial duration**: 9 weeks
- **Temperature**: 19º C
- **Salinity oscillation (9)**: randomly every 7 days
- **Feeding frequency:** twice a day
- **Diet (2):** seaweed diet (SW: *Gracilaria* sp. + *Uva* sp. + *Fucus* sp.) and control diet (without SW)
- **Parameters (4)**: Fish weight; Feed Conversion Ratio (FCR); Voluntary Feed Intake (VFI); Daily Growth Index (DGI)

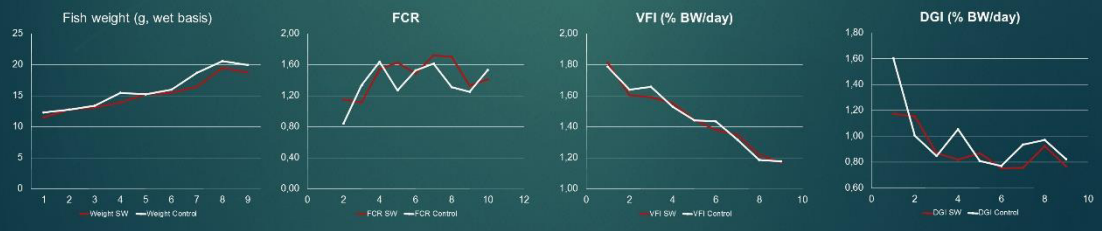
Weeks	1	2	3	4	5	6	7	8	9
Salinity (ppt)	35	13	17	15	30	12	18	26	24


Seabass fingerlings (10.5 ± 0.2 g)

12 tanks 6 tanks

Results

Under the experimental conditions, fish weight (11.88 to 20.03 g), FCR (1 to 1.67), VFI (1.18 to 1.80 % BW/day) and DGI (0.76 to 1.39 % BW/day) did not vary significantly ($p > 0.05$) between control and SW groups.



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Certifica-se que Gaspar Sensaim Mendes Filho
esteve presente no IJUP'16 – 9º *Encontro de Jovens Investigadores da Universidade do Porto*, que decorreu nos dias 17, 18 e 19 de fevereiro de 2016, na Fundação da Juventude – Palácio das Artes.

Pela Comissão Organizadora

Maria João Ramos

(A Vice-Reitora, Prof. Doutora Maria João Ramos)